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Studies of The PTEN Tumour Suppressor In Endometrial Cancer

Rachel Crosland

**A thesis submitted in partial fulfilment of the requirements of
Sheffield Hallam University
for the Degree of Doctor of Philosophy**

JANUARY 2004

ABSTRACT

Somatic mutations in the PTEN gene (Phosphatase and Tensin Homologue Deleted on Chromosome Ten) have been found in many types of cancer, but most frequently in cancer of the endometrium.

The PTEN gene encodes a D3' lipid phosphatase of the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3), which activates protein kinase B/Akt. Constitutive activation of Akt has been found in cells that lack functional PTEN, thus by dephosphorylating PIP3, this enzyme modulates several cellular functions e.g. proliferation, differentiation and migration.

The PTEN protein comprises a 403 amino acid, 55KDa protein which is present in the cytoplasm but has also been detected in the nucleus of some cells. The function of nuclear versus cytoplasmic PTEN has not yet been determined. Little is known about modulation of PTEN expression by molecules such as hormones and cytokines. It has been reported, however, that NGF, BDNF and vitamin D3 analogues can up-regulate PTEN. The steroid hormones oestrogen and progesterone have been proposed as mediators of PTEN transcription, since their expression in endometrium reflects the menstrual cycle. The role of TGF β 1 in PTEN expression of PTEN in human cells has been also investigated, but the results are contradictory. Compounds which stimulate up-regulation of PTEN represent potential anti-tumour therapies and therefore merit investigation. To further investigate the role of PTEN in endometrial cancer the following approaches were taken.

The effect of TGF- β 1 on two endometrial carcinoma cell lines, HEC-1B and Ishikawa were investigated. The cell lines were stimulated with TGF- β 1 in the presence or absence of serum, and changes in mRNA and protein levels of PTEN and other genes analysed by RT-PCR and Western blotting. The morphology, cell number and cell viability were also assessed. Modest up-regulation of PTEN mRNA was detected in both cell lines, but little change in protein levels was observed. In accordance with published data, TGF- β 1 suppressed the growth of, and changed the morphology of both cell lines.

To study PTEN sub-cellular localisation, full-length human PTEN cDNA was used in RT-PCR to generate a 1.2Kb fragment which was cloned into a green fluorescent protein expression vector pEGFP-N1 to create pRC-2. Sequencing of pRC-2 confirmed the in-frame cloning of wild-type PTEN. Lipid-based

transfection was used to transiently transfect HEC-1B, Ishikawa and Cos-7 cells. Strong perinuclear and cytoplasmic localisation was detected in these cell lines, and localisation to the endoplasmic reticulum was observed. Stimulation with TGF- β 1 and 17- β -estradiol had no discernable effect on sub-cellular localisation of PTEN in either HEC-1B or Ishikawa cell lines.

A mutational study was performed using a large repository of archival endometrial carcinomas and normal cervical controls. PCR was used to amplify PTEN exons 5 and 8 from extracted DNA and the fragments separated by single-strand conformation polymorphism (SSCP) analysis. A number of samples exhibiting bandshifts were detected in both exons.

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LIST OF ABBREVIATIONS

| | |
|--------------------------------|--|
| AA/aa | Amino Acid |
| Akt/PKB | Akt / Protein Kinase B |
| AR | Androgen Receptor |
| CBP | CREB Binding Protein |
| CDK | Cyclin Dependent Kinase |
| CK2 | Casein Kinase 2 |
| CM | Complete Medium |
| CMV-EGFP-N1 | Plasmid containing green fluorescent protein tag |
| DSP | Dual-Specificity Phosphatase |
| EC | Endometrial carcinoma |
| ECM | Extracellular Matrix |
| EGF(R) | Epidermal Growth Factor (Receptor) |
| ER | Endoplasmic Reticulum |
| ERK | Extracellular signal-Regulated Kinase |
| FAK | Focal Adhesion Kinase |
| FH / FOXO | Forkhead (Transcription Factor) |
| HEC-1B | Human Endometrial Carcinoma cell line-1B |
| Ig | Immunoglobulin |
| IGF(R) | Insulin-like Growth Factor (Receptor) |
| IKK | I κ B Kinase |
| IκB | Inhibitory unit of NF- κ B |
| ISK | Ishikawa cell line |
| JNK | c-Jun N-terminal kinase |
| Kb | Kilobase |
| KDa | KiloDalton |
| LF2K | Lipofectamine 2000 |
| MAPK | Mitogen Activated Protein Kinase |
| mTOR | Mammalian Target of Rapamycin |
| MDE | Mutation Detection Enhancement gel |
| NF-κB | Nuclear Factor Kappa-B |
| NLS | Nuclear Location Signal/Sequence |
| PAGE | Polyacrylamide gel electrophoresis |
| PEST | Proline-Glutamate-Serine-Threonine |

| | |
|--------------------------------|---|
| PCR | Polymerase chain reaction |
| PDGF(R) | Platelet-Derived Growth Factor (Receptor) |
| PDZ | Protein Dimerization Zipper |
| PI(3,4,5)P3 | Phosphatidylinositol 3,4,5-trisphosphate |
| PI(3,4)P2 | Phosphatidylinositol 3,4-bisphosphate |
| PI3-K | Phosphatidylinositol 3-kinase |
| PLC | Phospholipase C |
| pRC-2 | Clone containing full-length human PTEN cDNA |
| PTEN | Phosphatase and Tensin Homologue Deleted on Chromosome Ten |
| PTEN-2 | Murine testis-specific PTEN homologue |
| P-PTEN | Phospho-PTEN |
| PtdIns | Phosphatidylinositol |
| PTP | Protein Tyrosine Phosphatase |
| RT | Reverse Transcription |
| rTK | Receptor Tyrosine Kinase |
| SAPK | Stress-Activated Protein Kinase |
| Ser | Serine |
| SDS | Sodium dodecyl sulphate |
| SH | Src Homology domain |
| SSCP | Single Strand Conformation Polymorphism |
| TAE | Tris-acetate EDTA |
| TBE | Tris-borate EDTA |
| TEMED | N,N,N',N'-tetarmethylethylenediamine |
| TGF-β1 | Transforming Growth Factor-beta 1 |
| Thr | Threonine |
| TNF-α | Tumour Necrosis Factor-alpha |
| TPIP | TPTE and PTEN homologous inositol lipid phosphatase |
| TPTE | Transmembrane phosphatase and tensin himology |
| Tyr | Tyrosine |
| VEGF | Vascular Endothelial Growth Factor |

RESEARCH MEETINGS ATTENDED

British Cancer Research Meeting, 1-4th July 2001, Leeds, UK.

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Pathology 2003, 1-4th July 2003, Bristol, UK.

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CHAPTER 1

INTRODUCTION

1.1 Intracellular Signalling and Phosphatidylinositol 3-kinase (PI-3K)

The regulation of cell growth and death is crucial to the survival of all multicellular organisms. Environmental stimuli, such as growth factors, control the delicate balance between cellular life and death by producing the desired response from the target cell. Binding of a stimulating molecule to a transmembrane receptor is a common event, which activates transmission of the specific signal via the generation of intracellular second messengers such as cyclic AMP and phosphoinositols (PIs). Once activated these transient molecules elicit activation, usually by phosphorylation, of a downstream target. The signalling cascade may involve many molecules and be highly redundant, but the net effect includes the modulation of gene expression to exert control over cell growth and metabolism.

1.1.1 Signalling Through Receptor Tyrosine Kinases

The generation of intracellular signals can be achieved by the binding of extracellular growth factors or cytokines to specific receptor tyrosine kinases, which are activated by tyrosine phosphorylation. Ligand binding to a receptor tyrosine kinase, which can exhibit autoinhibition in the absence of ligand (Schlessinger 2003), causes rapid phosphorylation of the cytosolic domain which may induce receptor dimerisation. This can then be followed by the activation of G-proteins (Nystrom & Quon, 1999).

G-proteins are 3-subunit transmembrane complexes which bind GTP or GDP, and of which there are four subclasses. The G-protein is directly linked

to the enzyme which catalyses second messenger generation, for example by various isoforms of phospholipase C (PLC). Phosphorylation of the G-protein results in the activation of the enzyme, and for the PLCs in the generation of inositol-3,4,5-trisphosphate (IP3) and diacylglycerol (DAG) from phosphatidylinositol-4,5-bisphosphate (PI4,5P2). IP3 stimulates calcium release from the endoplasmic reticulum (ER), and DAG activates protein kinase C (PKC) to stimulate proliferation. In addition, DAG is phosphorylated by DAG-kinases to phosphatidic acid and transported to the ER, where it is combined with inositol to perpetuate the generation of phosphoinositides (Payrastre, et al, 2001 & Vazquez-Prado, et al 2003).

Signalling through receptor tyrosine kinases (rTKs) can also occur in a G-protein independent manner. Activation by ligand binding causes rapid dimerisation of receptors (eg for EGF and PDGF) or conformational changes in dimeric receptors, for example for insulin and IGF-1 (Hubbard, et al, 1998). This permits activation of the cytoplasmic tyrosine kinase domain by autophosphorylation of tyrosine residues in this region. An activated rTK is then capable of engaging a wide range of downstream molecules containing src homology domains (SH2), including growth factor receptor bound protein 2 (GRB-2) and phosphatidylinositol 3-kinase (PI3K). SH2 domains are characterised by binding specifically to phosphorylated tyrosine motifs and can therefore regulate the catalytic functions or localisation of their substrates (Baca, 2003).

Binding of the SH2 domain of PI3K, a heterodimer which is composed of a regulatory p85 and catalytic p110 subunit, causes a conformational change in p85. This change permits activation of p110 and localises the activated PI3K

to the plasma membrane for the 3' phosphorylation of phosphatidylinositols (PtdIns). These products are not cleaved by PLC and represent a distinct signal transduction mechanism (Vanhaesebroeck, et al, 1999c).

Convergent signalling pathways can be activated by binding of ligand to non-receptor tyrosine kinases, such as src and JAK (Janus kinase) as observed in B- and T-cell antigen receptor signalling (Stephens, et al., 1993). PI3K recruitment and activation via p85 may occur due to these kinases binding to intermediates containing src homology-3 (SH3) domains. SH3 domains bind proline rich motifs on both upstream and downstream molecules simultaneously and mediate multiple protein-protein interactions (Jiang & Qui 2003). The overall effect, as with activation of rTKs, is the localisation of active PI3K enzyme to the plasma membrane.

Finally, as signalling is a redundant mechanism, PI3K may also be activated by the Ras family members which initiate the mitogen activated protein kinase (MAPK) pathways (Vanhaesebroeck, et al, 1997). Ras, like PI3K, is a common effector of pathways activated by many growth factor receptors and binds GTP with high affinity when activated. PI3K recruitment by GTP-Ras occurs via direct interaction with the p110 subunit, restoring catalytic function without binding to p85.

1.1.2 The PI-3K Pathway Intracellular Signal Transducers

The activation and recruitment of PI3K to the plasma membrane is the starting point of lipid-mediated signal transduction via a serine/threonine phosphorylation cascade. Three distinct classes of PI3Ks have been identified based upon p110 lipid substrate specificity and mechanism of regulation, with Class IA being most commonly associated with signalling through receptor tyrosine kinases and the only class to activate protein kinase B (PKB) (Vanhaesebroeck et al., 1999 & Fruman, et al., 1998). All mammalian Class I PI3Ks exhibit inhibition by low levels of the small cell-permeable compounds wortmannin and LY294002, which are unrelated structurally but which bind covalently to p110 (Wymann, et al, 1996) or competitively inhibit ATP binding (Vlahos, et al, 1994) . The PI3K class and associated inositol lipid products are shown in Table 1.

| PI3K Class | Features | Substrates | Inositol products | Downstream Roles |
|------------|--------------------------------|------------|-------------------|--|
| I | Activated by rTKs & G-proteins | PI | PI3P | Proliferation Vesicular Trafficking Cytoskeletal Rearrangements Gene Expression |
| | | PI4P | PI3,4,5P3 | |
| | | PI4,5P2 | PI3,4,P2 | |
| II | Possess C2 domain | PI | PI3P | Vesicular Transport from Golgi Gene Expression |
| | | PI4P | PI3,4P2 | |
| III | Forms heterodimers | PI | PI3P | |

Table 1 PI3K Classes, their products and cellular roles

The catalytic p110 subunit exists as isoforms α , β and δ which associate with regulatory/adaptor p85 isoforms α , β and γ (Hu *et al.*, 1993) to achieve lipid kinase activity. The p110 γ isoform, however, binds an adaptor protein p101 and is activated by the $\beta\gamma$ subunits of G proteins, as is the p110 β subunit

(Stoyanov et al., 1995 & Kurosu et al., 1997). These isoforms are expressed in a tissue specific manner and have distinct roles in transducing signals generated through distinct receptors. For example, p110 α transduces PDGF-derived signals and p110 β is the transducing factor for insulin (Hooshmand-Rad, *et al.*, 2000). p110 δ is predominantly expressed in mature B and T cells and mediates antigen-receptor signalling (Zhang, Vanhaesebroeck & Rittenhouse, 2002), whereas p110 γ is required for T-cell development and CD4/CD8 lineage commitment (Rodriguez-Borlando, et al, 2003).

It is thought that the isoform-specific protein kinase activity of p110 subunits towards themselves (by autophosphorylation) and their adaptor proteins contributes to the functional specificity of PI3Ks (Vanhaesebroeck et al., 1999b and c). The isoforms of p85 mentioned above contain a range of domains (Cohen *et al.*, 1995) including multiple SH2/3 and a region for binding p110 isoforms to regulate kinase activity (Klippel *et al.*, 1994). Recent work by Okkenhaugik & Vanhaesebroeck (2001) also indicates a regulatory role for p85 in actin cytoskeleton rearrangement.

Membrane localised PI3K specifically phosphorylates the D3 position of the inositol ring of phosphatidylinositides to generate phosphatidylinositol-3,4-bisphosphate (PI3,4P2) and phosphatidylinositol-3,4,5-trisphosphate (PI3,4,5P3, also written as PIP3). These PtdIns are almost absent unless the cell is stimulated by a growth factor, unlike other cellular PtdIns which are constitutively expressed (Carpenter et al., 1996). These include phosphatidylinositol-4,5-bisphosphate (PI4,5P2), which is involved in the PLC/DAG pathway as previously mentioned, and phosphatidylinositol-3-phosphate (PI3P) which has a role in membrane trafficking via the

recruitment of proteins containing FYVE domains and in receptor sorting (Leevers, et al., 1999 & Petiot, et al, 2003). PI3Ks generate molecules which activate many downstream effectors, (discussed in detail below), the dysregulation of which is implicated in pathological disorders such as cancer, allergy and cardiovascular diseases.

PI3Ks are now the target of novel drugs to selectively inhibit specific isoforms of the kinase, in the hope of eliminating disease without abolishing entire signalling pathways (Arcaro, et al 2002 & Wymann, M. P., et al 2003). Recently, the p110 δ/γ subunits of PI3K have been found to be essential non-redundant mediators of immune function, with implications for treating autoimmunity and transplant rejection (Okkenhaug & Vanhaesebroeck, 2003)

1.1.3 Downstream Targets of PI3K Activation

Membrane associated PI3,4P2 and PI3,4,5P3 generated by Class I PI3K are capable of activating PKB by binding to downstream target molecules via Pleckstrin homology (PH) domains, some of which target the inositol lipids and their head groups with high affinity (Vazquez & Sellers, 2000).

Proteins containing PH domains for PI3,4P2 and PI3,4,5P3 in this pathway include the Ser/Thr kinases PKB, also known as Akt, and PDK-1 (phosphoinositide-dependant kinase-1). Akt/PKB exists as α , β and γ isoforms which are widely expressed in tissues, consisting of an N-terminal PH domain, a kinase domain and a C-terminal regulatory tail (Wymann, et al, 1998). These latter two domains contain ser/thr sites (Ser473 and Thr308) which must be phosphorylated to fully activate the kinase (Alessi, et al, 1996a). The PH domain of Akt/PKB α preferentially binds to PI3,4,5P3,

causing its translocation from the cytoplasm to the membrane and a conformational change. This change exposes an activation loop containing Thr308 and Ser473, (or equivalent residues in the other isoforms), and initiates the phosphorylation of Thr-308 by PDK-1.

The mechanism of Ser473 phosphorylation is thought to occur through integrin-linked kinases functioning as adaptors to recruit a Ser473 kinase (Lynch et al., 1999), or by other rTKs (eg for IGF) by virtue of an interaction between PDK-1 and the C-terminal fragment of PRK-2 (protein kinase C-related kinase-2). This fragment, termed PDK1-interacting fragment (PIF) has been observed to convert PDK1 in a manner which permits it to phosphorylate Ser473 of Akt/PKB. Recent findings implicate Ser473, but not Thr308, as being the target residue for modulation of Akt/PKB activity (Wan & Helman, 2003, Dery, et al 2003, & Woods, et al, 2003). The interaction with PIF also causes a three-fold activation of PDK-1 by PI3,4,5P3 or PI3,4P2 but not PI4,5P2 (Balendran et al., 1999a). As PDK-1 complexes with other PKC family members, these interactions may form the basis by which modulation of PDK-1 towards Akt/PKB is achieved (Pullen et al., 1998, Le Good et al., 1998, & Balendran, et al, 1999b).

In addition to Akt/PKB, PDK-1 is also capable of activating p70-S6 kinase to initiate cell cycle progression via increased translation of immediate-early genes such as cyclin D (Takuwa, et al, 1999) and E2F transcription factor (Brennan, et al, 1999). Some components of the PI3K pathway demonstrate the ability to travel between the nucleus and cytoplasm, including PI3K, Akt/PKB and insulin signal mediators. Recently, PDK-1 has also been verified as existing in the nucleus, and this may represent a mechanism for

sequestering the protein to prevent it from participation in the cytosolic signalling cascade (Lim, M. A., et al 2003).

1.1.4 Downstream Targets of Akt

Once activated, Akt/PKB is capable of phosphorylating serine and threonine residues in small peptides *in vitro* containing the motif RXRXXS/T*, where X is any amino acid and * is phenylalanine or leucine (Alessi et al., 1996). Many of Akt/PKBs substrates are inactivated by this phosphorylation, at least *in vitro*, and therefore include pro-apoptotic factors such as BAD, Caspase 9, GS3K β and Forkhead transcription factors (Downward, 1998). Overexpression of Akt/PKB isoforms is anti-apoptotic and delays cell death, as observed in cancer cell lines representing pancreas (Cheng et al., 1996), breast (Nakatani et al., 1999) and ovary (Cheng, et al., 1992). An overview of the possible substrates of Akt/PKB are illustrated in Figure 1.

Akt/PKB Signaling Pathways

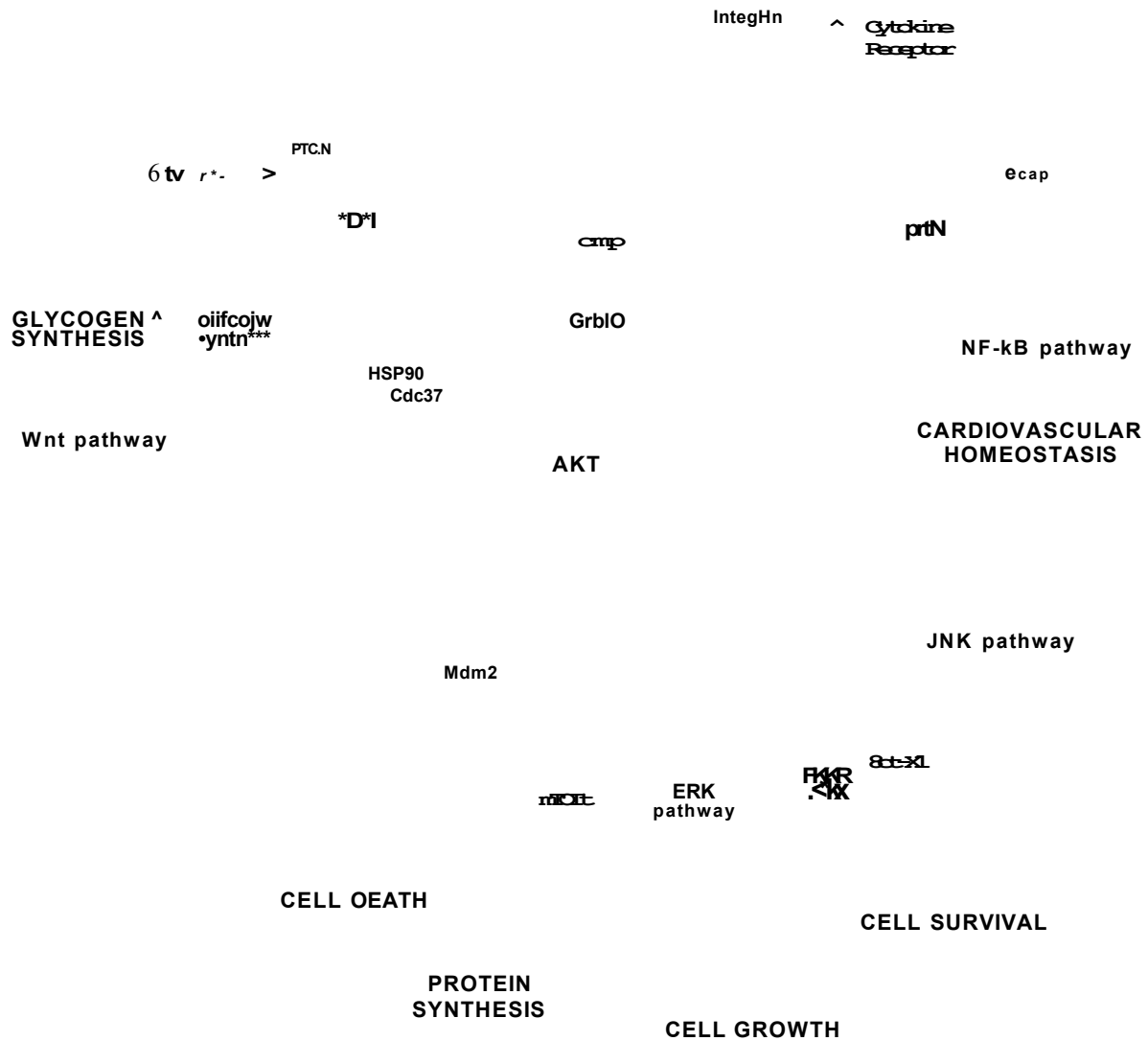


Figure 1 Akt/PKB Pathways Figure legends overleaf.

Taken from www.cellsignal.com

BAD

The BAD protein (Bcl-2/Bcl-X_L-antagonist, causing cell death) exists as a heterodimer with either Bcl-2 or Bcl-X_L, which are anti-apoptotic, and inhibits their function. When phosphorylated on Ser136 or Ser112, BAD is bound by 14-3-3 adaptor proteins and releases Bcl-2/Bcl-X_L which inhibit apoptosis. (Downward, 1999). Not all cell types express BAD, however, and other mechanisms of cell survival regulation exist independently of BAD phosphorylation and Akt/PKB activation (Hinton & Welham, 1999).

Caspase-9

Caspase-9 is an important protease in the initiation of the terminal apoptotic cascade and directly cleaves and activates the executioner molecule caspase-3 (Alnemri, 1999 and Wolf, et al, 1999). Akt/PKB has been shown to phosphorylate and inactivate human caspase-9 (Cardone, et al, 1998 and Takeuchi & Ito, 2003) but the phosphorylated residue is not conserved in rat, mouse or monkey homologues (Fujita, et al, 1999). There is also evidence that Akt/PKB may intervene at an earlier stage in the apoptotic cascade, prior to caspase-9 activation, by stabilising the mitochondrial membrane (Kennedy, et al, 1999).

ASK1

The apoptosis signal-regulating kinase-1 (ASK1) is a recently discovered substrate of Akt/PKB. ASK1 is a mitogen-activated protein kinase kinase kinase (MAPK kinase kinase) which acts upstream of p38 (Liao & Hung, 2003) and c-Jun N-terminal kinase (JNK) pathways, which are activated by

stress and pro-inflammatory cytokines to induce apoptosis. Work by Kim, et al (2001) demonstrated phosphorylation of ASK1 by Akt/PKB at Ser83 in a consensus region similar to other Akt/PKB substrates. The two proteins also co-immunoprecipitated in a manner observed in Akt/PKB-BAD interactions (Datta, et al, 1997) and could associate constitutively, but activation of Akt/PKB was necessary for ASK1 phosphorylation.

Modulation of ASK1 by Akt/PKB phosphorylation results in an inhibition of JNK and p38, which initiate and modify transcription through a range of transcription factors including ATF-2 and AP-1. These factors promote apoptosis via expression of Bcl homologues such as Bax and Bak, and may therefore indicate a transcription-based regulatory mechanism for Akt/PKB in apoptosis (Kyriakis, 1999 and Tibbles & Woodgett, 1999). Recently, Akt/PKB has been shown to bind to JNK interacting protein-1 (JIP1) in mouse primary neurones. This interaction inhibited JIP1-mediated activation of JNK pathway kinases and thus decreased apoptosis (Kim, et al 2002).

Additional targets in the stress-activated protein kinase (SAPK) pathways may include the Rac1 G-protein which activates the JNK pathway (Kwon, et al, 2000). The involvement of Akt/PKB is complex, and illustrates the cross-talk between lipid signalling and MAP kinase cascades. This is further complicated by observation that although a single stimulus may activate multiple pathways, not all of these pathways are required to elicit the appropriate cellular response (Kirit, et al, 2000). There is additional evidence that Akt/PKB may phosphorylate multiple targets simultaneously and act as a central regulator of multiple signalling pathways (Kim, et al, 2001).

Forkhead Transcription Factors

Akt/PKB is known to interfere with apoptosis at the transcription level via two mechanisms, the first of which involves members of the Forkhead (FH) transcription factor family. Three members of this superfamily, FKHR, FKHRL1 and AFX have been shown to be phosphorylated by Akt/PKB on three residues (Kops, et al, 1999, Rena et al, 1999 and Brunet et al, 1999). During cellular stimulation, FH transcription factors are translocated from their normal location in the nucleus to the cytoplasm (Brunet, et al, 1999). It is thought that phosphorylation by Akt/PKB prevents FH target gene transcription by promoting nuclear export of the FH proteins to the cytosol where they are sequestered by 14-3-3 adaptor proteins, thus preventing access to their pro-apoptotic target genes (Guo, et al, 1999 and Biggs, et al, 1999).

Recent findings have implicated Akt/PKB-regulated FH transcription factors, now termed FOXO (Burgering & Medema, 2003) in the control of expression of proteins directly involved in the cell cycle. A clone of HL60 human leukaemia cells with constitutive Akt/PKB (HL60AR) displayed higher levels of phosphorylated FH transcription factors Fox01 (FKHR) and Fox03 (FKHRL1) than parental cells. These factors were permanently restricted to the cytoplasm resulting in increased proliferation. The cyclin-dependent kinase (CDK) inhibitor p27^{Kip1} protein was observed to be both decreased and hyperphosphorylated, levels of cyclin D1 were increased as were the activities of cdk2, 4 and 6, and the retinoblastoma (Rb) protein was also phosphorylated (Cappellini, et al, 2003). These findings indicate control of the cell cycle by Akt/PKB via nuclear exclusion of FOXO transcription factors,

and similar results have been observed in hepatocytes (Wolfrum, et al, 2003) and glioma cells (Ciechomska, et al, 2003). FOXO factors are capable of both inhibiting proliferation and initiating apoptosis via distinct mechanisms, and could be especially important in cells of the immune system (Birkenkamp & Coffey, 2003).

NF- κ B & I κ B kinases (IKK)

The second anti-apoptotic transcription-based mechanism employed by Akt/PKB involves the transcription factor nuclear factor κ -B (NF- κ B), which is at the endpoint of many signalling pathways. NF- κ B exists in the cytoplasm bound to its inhibitor, I κ B, until it is phosphorylated by I κ B kinases (IKKs) and degraded, allowing NF- κ B to translocate to the nucleus (Mercie, et al, 1998 and Dent et al, 2003). Here, transcription of anti-apoptotic genes occurs, including inhibitor-of-apoptosis (IAP) proteins c-IAP1 and 2 (Wang, 1998). Akt/PKB has previously been shown to associate with IKKs (Kane, et al, 1999) and to mediate IKK α phosphorylation at Thr23 (Ozes, et al, 1999). As with human caspase-9, the predicted phosphorylation site on IKK α does not lie within the optimal consensus sequence for Akt/PKB (Ozes et al, 1999), and may involve additional mediators such as protein kinase CK2 (Romieu-Mourez, et al, 2002) which is upregulated in some tumours.

GSK3 β & Insulin Signalling

Akt/PKB appears to have a crucial role in mediating insulin signalling, as over-expression of activated Akt/PKB elicits the same effects as insulin in responsive cells (Barthel, et al, 1999), and PI3K inhibitors prevent insulin signalling (Shepherd et al, 1998). Although the exact mechanism is unknown, Akt/PKB has been shown to phosphorylate and inactivate glycogen synthase kinase-3 β on Ser9 (Grimes & Jope, 2001). GSK3 β phosphorylates cyclin D1 on Thr286 (Diehl, et al, 1998), causing its degradation by the proteasome and preventing G1-S transition of the cell cycle. Phosphorylation and inhibition of GSK3 β would therefore allow the accumulation of cyclin D1 and progression of the cell cycle.

Other targets of Akt/PKB downstream of the insulin receptor include insulin receptor substrates -1&-2 (IRS-1&-2), mammalian target of rapamycin (mTOR) and phosphodiesterase-3B (PDE-3B). IRS-1 is tyrosine-phosphorylated and activated by the insulin receptor allowing association with p85 α of PI3K. Akt/PKB appears to positively regulate IRS-1 by phosphorylation of one or more of four possible Ser residues in a feedback mechanism which protects active IRS-1 from dephosphorylation by protein tyrosine phosphatases (Paz et al, 1999). Recently Ser302 has been shown to be one of the residues phosphorylated (Giraud, et al, 2003 and Johnston, Pirola & Van Obberghen, 2003). IRS-2 is modulated through both mRNA transcription and protein regulation, but the exact mechanism is unknown (Hirashima, et al, 2003).

The mTOR protein is critical in the integration of signals which detect nutrient availability and allow cell cycle progression, and is inhibited by rapamycin

and its analogues. (Abraham, 2002). mTOR is a downstream target of Akt/PKB and PDK-1 (Section 1.1.3) which activate the protein and initiate an increase in the synthesis of proteins required for cell cycle progression such as cyclin D1. This pathway has been found to be elevated in several cancer cell lines including pancreas, lung and prostate, and inhibitors of mTOR are potential anti-tumour agents (Gera, et al, 2003 and Gao, et al, 2003).

In response to insulin binding, Akt/PKB also phosphorylates and activates PDE-3B on Ser273. PDE-3B is present in adipocytes and pancreatic β cells where it controls cellular levels of the second messengers cAMP and cGMP, and prevents lipolysis (Kitamura et al, 1999), and possibly promotes cell survival and cell cycle progression (Ahmad et al, 2000)

Endothelial Nitric Oxide Synthase (eNOS)

eNOS is produced by endothelial cells in response to VEGF and shear stress and activated by Akt/PKB, which can phosphorylate eNOS on Ser1177 (Dimmeler, et al, 1999). Recently, insulin has also been shown to regulate eNOS expression, but not activity, via increasing the binding of the transcription factors AP-1 and Sp1 (Fisslthaler, et al, 2003). Increased eNOS expression has been implicated in the onset of diabetes and hypertension (Pieper, 1998). Nitric oxide (NO) production by eNOS causes vasodilation and can modulate apoptosis, exerting both pro- and anti-apoptotic effects depending on concentration and cell type (Pervin, Singh, & Chaudhuri, 2003). NO produced in response to VEGF increases angiogenesis, and may be a mechanism by which tumours increase their blood supply.

BRCA1

The breast cancer susceptibility gene BRCA1 encodes a nuclear phosphoprotein that acts as a tumour suppressor and is inactivated in breast and ovarian cancers. BRCA1 is regulated through the cell cycle by CDK2 and also in response to DNA damage by Cds1, and its phosphorylation prevents its activity in attenuating the cell cycle (Lee, et al, 2000). The growth factor family of heregulins have been implicated in the survival of breast cancer cell lines, and are known to inactivate BRCA1 via Akt/PKB-mediated phosphorylation of Thr509 (Altioik, et al, 1999). Furthermore, inactivation of BRCA1 has been shown to be enhanced by the extracellular matrix (ECM), which often interacts abnormally with tumour cells to promote invasion of surrounding tissue (Miralem & Avraham, 2003).

In addition to the substrates discussed in detail above, new targets of Akt/PKB are continually adding to the repertoire of this kinase in regulating cell survival via multiple pathways. Recent discoveries include the negative regulation of Elk-1 mRNA translation, a transcription factor downstream of MAPK signalling (Figueroa & Vojtek, 2003) and the MAP kinase activator Raf in some cell lines (Zimmerman & Moelling, 1999). Direct phosphorylation and inhibition of the CDK inhibitor p27^{kip1} at Thr198 and Thr157 by Akt/PKB has been reported, preventing constraint of G1 progression by relocalising p27^{kip1} to cytoplasmic 14-3-3 proteins (Shin, et al, 2002 and Fujita, Sato & Tsururo, 2003). Akt/PKB also causes IGF-induced phosphorylation of WNK1 (with no K [lysine] protein kinase-1) on Thr60, a kinase that is mutated in an inherited hypertensive disorder (Vitari, et al, 2003).

1.2 PTEN Tumour Suppressor & PI3K Signalling

The importance of regulating intracellular signalling is dramatically illustrated by the many disorders resulting from mutation or otherwise inactivation of the genes which govern these pathways. The most prominent pathological process is cancer, described as a disease of the cell cycle, whose many forms provide insight into the complexities of cell life and death. Many genes have been characterised in the role of guardian of the cell cycle, termed proto-oncogenes and tumour suppressors. Of the latter p53 and Rb are perhaps the best understood, but recently a new tumour suppressor was isolated. Called PTEN, this gene rivals p53 for the most commonly mutated gene in human cancer.

1.2.1 PTEN - A Tumour Suppressor Discovered Twice

In 1997 a novel tumour suppressor gene was simultaneously discovered by two groups focussing on the high incidence of mutations and allelic loss on human chromosome 10. The gene, which localised to the long q arm at locus 23.3, was named PTEN (Phosphatase and Tensin Homologue Deleted on Chromosome Ten, (Li, et al, 1997) and MMAC-1 (Mutated in Multiple Advanced Cancers-1, Steck, et al, 1997) respectively. A third group submitted their findings shortly afterwards, named the gene TEP-1 (TGF- β Regulated and Epithelial Cell Enriched Phosphatase-1, (Li & Sun, 1997). As the names suggest, analysis of PTEN cDNA revealed a sequence coding for a 403 amino acid 55KDa protein of 9 distinct exons, with N-terminal homology to the cytoskeletal proteins auxilin and tensin. The sequence also contained a highly conserved catalytic motif IHCKAGXXRTG at residues

122-133 (Denu & Dixon, 1995 and Tonks & Neel, 1996), which is found in protein tyrosine and dual-specificity phosphatases (PTP and DSP). DSPs, such as yeast CDC14 (Li, et al, 1997) and PTPs, such as the yeast PTEN homologue CDC25 (Flint, et al, 1997 and Li & Sun, 1997), are components of signal transduction and the cell cycle regulatory machinery in *S.cerevisiae*. The presence of the phosphatase motif therefore indicated a possibly crucial role for PTEN as a protein tyrosine phosphatase in cell cycle modulation, and therefore in the development of cancer.

1.2.2 PTEN Structure and Function

1.2.2.1 PTEN Gene and mRNA Structure

Although the cDNA sequence for PTEN (illustrated in Appendix II) has been known for some years, it is only recently that the 5' promoter region upstream of the PTEN genomic sequence has been described. A lack of classical promoter motifs, such as TATA boxes, added to the question of how PTEN mRNA was transcribed. Sheng, et al (2002) investigated this problem by constructing various minimal promoter cassettes from a 2212bp genomic fragment previously cloned by Steck, et al, (1998) named BAC-46B12. This fragment contained a portion of the 5' untranslated region of exon1 and a 5' portion of the flanking PTEN sequence. The investigation revealed nine potential transcription start sites from -958 to -821 prior to the initiation codon ATG (+1) in a 599bp fragment termed the minimal promoter region. Of these, three sites at -972, -869 and -837 were identified as potential major transcription initiation sites, with the 137bp prior to +1 being the absolute

minimum required for transcription. The minimal promoter region is shown schematically in Figure 2.

Additional features uncovered by Sheng, et al (2002) included several consensus sites for binding transcription factors including Sp1 and Egr-1 (between -958 and -821), which may have opposing effects on promoter activity. The cyclin inhibitor p15^{INK4B} is upregulated by TGF- β 1 via Sp1 (Feng & Derynck, 2000), suggesting that Sp1 may play a role in PTEN expression. The presence of a p53 binding site was investigated but the group found that p53 caused only a modest increase in PTEN expression. The relationship between p53 and PTEN is discussed in detail further in this section.

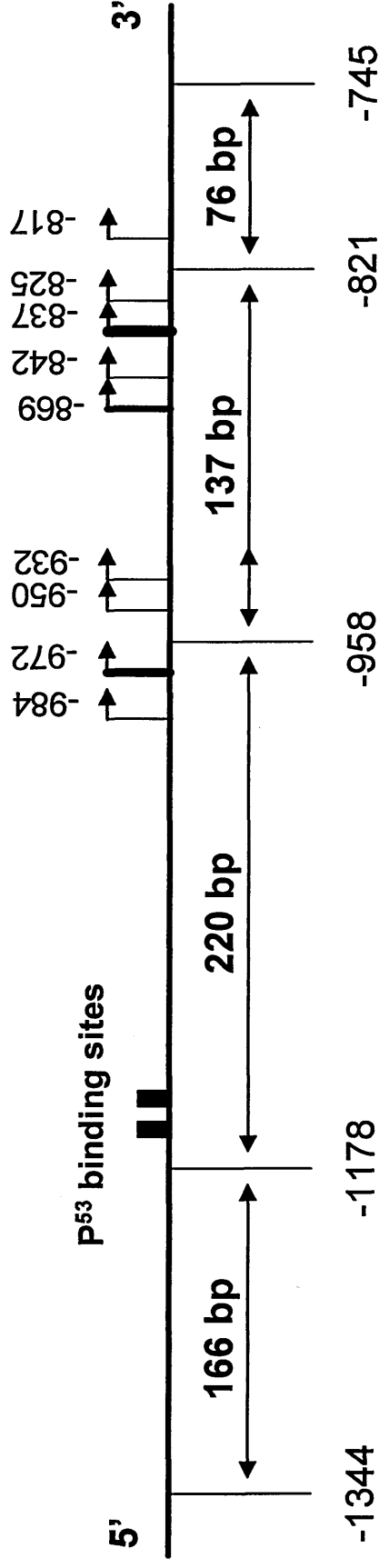


Figure 2 PTEN minimal promoter region

PTEN minimal promoter region showing nine transcription start sites. Arrowed bars represent transcription start sites relative to ATG(+1). Bold arrows show potential major transcription start sites, with -837 being the most common. The minimum promoter region from -958 to -821 contains six of the nine initiation sites and includes two Sp1 sites flanking an Egr-1 site. Adapted from Sheng, et al, 2002.

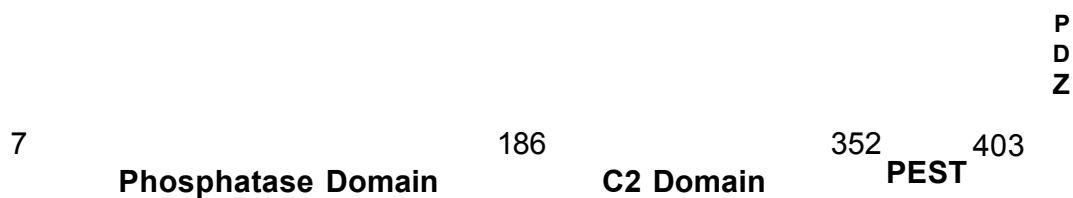
1.2.2.2 PTEN Protein: A Structure-Function Relationship

As previously mentioned, analysis of the PTEN sequence revealed an N-terminal phosphatase domain, and also C-terminal putative phosphorylation sites. Notably, the protein lacked a nuclear location sequence, and appeared to be cytoplasmically located. The determination of the crystal structure of PTEN by Lee, et al, (1999) gave greater insight into the functional aspects of this protein. The resolved structure is shown in Figure 3.

The structure revealed several interesting features of PTEN. Firstly, the PTP-like N-terminal domain forms part of an enlarged active site, and the C-terminus contains a C2 domain. Calcium independent C2 domains bind lipid vesicles *in vitro*, and the presence of a membrane-binding motif in addition to the active site is common in enzymes against inositol phospholipids substrates (Lee, et al 1999). A PDZ motif found in protein-protein interaction domains (Kocher, et al 2003) was located in the C-terminus, as were two PEST sequences which target proteins for degradation (Rogers, et al 1986). The domains are shown in cartoon form in Figure 3.

Initial studies to determine the physiological substrate(s) of PTEN indicated that phosphoproteins and peptides were not major targets, due to weak activity of the enzyme. Highly acidic peptides did undergo dephosphorylation by PTEN *in vitro* (Myers, et al, 1997 & Maehama & Dixon, 1998), but the major cellular substrate was found to be PI3,4,5P3 which is dephosphorylated to PI3,4P2. PTEN was therefore characterised as a lipid phosphatase, and its connection to the PI3K pathway was established (Myers, et al, 1998, Maehama & Dixon, 1999 & Stambolic, et al, 1998).

A



B

Figure 3 The Crystal Structure of PTEN

A .The structure of PTEN as determined by Lee, J. O., et al (1999), PDB 1DR5. The N-terminus is composed of p-sheets surrounded by a-helices and the C-terminus is composed of p-sheets. The catalytic site in the phosphatase domain is shown as red ball-and-stick area. **B**. Cartoon representation of PTEN. N-terminal phosphatase domain (aa 1-185 & catalytic motif 123-130), C-terminal domain (186-403) with C2 (186-351), PEST (350-375 & 379-396) and PDZ. Phosphorylation sites for CK2 (S380, T382 & T383) shown as a circled P.

In light of its substrate PI3,4,5P3, the features of the PTEN protein and their relevance to possible function are discussed below.

C-Terminal Domain

This domain contains C2, PDZ and PEST domains found in many other signal transducing molecules. The C2 domain, as previously mentioned, is associated with phospholipids and membrane binding. The C2 domain of PTEN does bind to phospholipid membranes *in vitro* (Rizo & Sudhof, 1998 & Hurley & Meyer, 2001) but lacks the acidic residues for Ca^{2+} binding seen in Ca^{2+} -dependant lipid phosphatases (Hurley, et al 2000). Functionally, mutations in this domain dramatically reduce lipid binding and cellular levels of PI3,4,5P3 are not reduced (Georgescu, et al, 2000 & Leslie, et al, 2001). Many mutation studies have shown that the minimal catalytic unit consists of the phosphatase and C2 units. Removal of a few bases in the C2 region is sufficient to disrupt all phosphatase activity, even against soluble PI3,4,5P3. Mutations in the C-terminal tail distal to the C2 domain however have little effect on catalysis (Georgescu, et al, 1999 & Tolkacheva & Chan, 2000.) It is thought that the C2 domain may not only allow PTEN to associate with membranes, but also to ensure the correct orientation of the active site relative to PI3,4,5P3 (Georgescu, et al, 2000).

The PDZ motif has been observed to affect the stability but not the activity of PTEN against lipid substrates (Georgescu, et al, 1999), but deletion of the PDZ disrupts cell spreading (Leslie, et al 2001). Work by Wu, et al, 2000a & Wu, et al, 2000b demonstrated a PDZ-specific interaction between PTEN and MAGI-2 and -3 (membrane-associated guanylate kinase inverted)

scaffold proteins. This interaction enhanced the ability of very low levels of PTEN to act on its substrate, suggesting such interactions may have a role in regulating PTEN activity and stability.

The two PEST sequences are also apparently required for stabilising the PTEN protein. Deletion of these motifs can cause a decrease in expression of the protein, but can also increase phosphatase activity of some C2 domain mutants (Georgescu, et al, 1999 & 2000). When phosphorylated the second PEST sequence can decrease PTEN activity (Vazquez, et al, 2000), suggesting that modifications to the PEST sequences can alleviate abnormal C2 mutations. Possible conformational changes may occur to correct the function of C2 mutants in a manner observed for p53 mutants (Foster, et al, 1999).

Finally, the C-terminus also contains several phosphorylation sites located in the last 50 amino acid "tail" of exons 8 and 9. These sites may be essential for PTEN stability and have a role in regulating cellular levels and activity of the protein. Phosphorylation of the C-terminal tail has been observed by protein kinase CK2 (Torres & Pulido, 2001) and is discussed further in this section.

N-Terminal Domain

The N-terminal domain contains the catalytic phosphatase core with the conserved motif residing in exon 5 at amino acids 123-130 (see Figure 3). Although similar to other protein phosphatases (PTPases and DSPs), the active site of PTEN is somewhat larger. For example, the active cleft of PTP1B is narrow and deep whereas that of the DSP VHR is shallower

(Tonks & Neel, 2001). The enlarged active site of PTEN is deep but also broad to accommodate the large inositol ring headgroup, and highly basic reflecting its preference for acid substrates (Lee, et al, 1999). Mutations are common in this region with ~30% of somatic and germline changes affecting exon 5. Mutations, and their associated diseases, are discussed in Section 1.5. Recently, the N-terminus has been shown to be involved in membrane targeting, and along with the C2 domain may ensure correct orientation of PTEN relative to its acidic substrate. Moreover, disruption of the N-terminal motif prevents catalysis of substrate *in vivo*, which can be restored by adding a myristoylation signal which enables targeting to the plasma membrane (Walker, et al, 2004).

Nuclear Localisation

As PTEN targets PI3,4,5,P3 it is ubiquitously expressed in the cytoplasm and has no nuclear location sequence (repeated positive aa flanked by proline residues), therefore little study was allocated to nuclear PTEN. Now, however, several immunohistochemical studies have reported PTEN in the nucleus of normal cells, with a shift to cytoplasmic localisation in corresponding tumour cells (Perren, et al 2000, Gimm, et al 2000, Tachibana, et al 2002 & Whiteman, et al 2002). Nuclear PTEN has been associated with G₀-G₁ in MCF-7 breast carcinoma cells, whereas the protein accumulates in the cytoplasm during S-phase, suggesting PTEN may directly regulate the cell cycle in the nucleus (Ginn-Pease & Eng, 2003). Differential localisation of PTEN in immature, mature and differentiating neural cells also indicates a role for nuclear PTEN in CNS development (Lachyankar, et al 2000).

Regulation of Protein Activity

Phosphorylation of the C2 Domain

As previously mentioned the 50 amino acid C-terminal 'tail' of PTEN protein contains several phosphorylation sites. Ser380, Thr382 and Thr383 are required to be phosphorylated to stabilise the protein, but this stabilisation may also reduce activity (Vazquez, et al 2000) and association with PDZ proteins (Adey, et al 2000). Constitutive P-PTEN has been observed in acute myeloid leukaemias and is associated with advancement of the disease and poor clinical outcome (Cheong, et al 2003). The protein kinase CK2 has been shown to phosphorylate Ser370, 380 and 385, and Thr383 and this appears to prevent degradation by the proteasome, thus influencing PTEN protein levels. (Torres & Pulido, 2001). This has very recently been investigated *in vivo* using cilostazol, a 3-phosphodiesterase inhibitor used as an anti-platelet drug which is neuroprotective. This drug decreased CK2-mediated PTEN phosphorylation in both ischemic rat brains (Lee, J. H., et al 2003) and SK-N-SH neuroblastoma cells (Kim, et 2003b), thus permitting degradation of PTEN and activation of Akt/PKB. However, mechanisms controlling the degree to which PTEN is phosphorylated are unknown.

Vazquez, et al (2001) have proposed a mechanism in which phosphorylation of the tail causes a conformational change from 'open' to 'closed' and prevents the recruitment of PTEN into protein complexes by masking the PDZ domain. The recruitment of PTEN into the protein complex may permit targeting to the membrane to bind PI3,4,5P3, therefore phosphorylation of the tail would effectively inhibit the lipid phosphatase function of PTEN. A second proposal by Das, et al (2003) suggests that phosphorylation of the tail

does not block the PDZ domain, but interferes with the electrostatic membrane binding capability of the protein. By blocking membrane docking, phospho-PTEN is not degraded, but it is still not clear how even the membrane-bound form is degraded or whether a phosphatase exists to dephosphorylate PTEN.

Another phosphorylation-dependant mechanism of PTEN regulation may be via Src family kinases. Lu, et al (2003) demonstrated that Src family kinases, but not purified Src or Lck, can tyrosine-phosphorylate PTEN, suggesting an intermediary protein is involved. The 5'-phospholipid phosphatase SHP-1 can regulate Src kinases and dephosphorylate many Src targets (Somani, et al, 1997 & Cuevas, et al, 1999), and can regulate PI3,4,5P3 via PI3K (Freeburn, et al, 2002 & Lu, et al 2003). It is possible therefore that SHP-1 may restore the function of PTEN by acting as a PTEN phosphatase (Lu, et al 2003).

Recently, fluorescent microscopy has indicated that nuclear PTEN in VCSM cells is phosphorylated and its activity reduced, whilst the 5'-phospholipid phosphatase SHIP-2 is active. The presence of nuclear PI4,5P2 and PI3,4,5P3 has been reported in many cell lines, but their role in the nucleus is unclear (Deleris, et al 2003).

CK2 phosphorylation also appears to protect PTEN from cleavage by TNF α -activated caspase-3, which also targets MAGI-2 scaffold proteins to which PTEN has been shown to associate with. Torres, et al, (2003) have identified several aspartate residues in the C2 domain of PTEN which are targeted by caspase-3, and these sites overlap with the CK2 phosphorylation sites in this region. The group demonstrated that phosphorylation by CK2 blocks caspase-3 cleavage, and may therefore protect PTEN from cleavage. During

apoptosis, cleavage of the C2 domain could change the interaction of PTEN with structural and regulatory proteins, such as MAGIs, and vault proteins which are involved in nuclear-cytoplasmic transport (Yu, et al 2002).

Inhibition of the Catalytic site

A study analysing the effect of oxidative stress on PTEN levels has revealed that reactive oxygen species (ROS), which are produced as a by-product of metabolism, by macrophages, and through growth factor signalling (Meng, et al 2002), could decrease PTEN activity (Leslie, et al 2003). ROS have previously been shown to inactivate and regulate PTPs by oxidising a critical cysteine in the active site (Tonks & Neel, 2001). These enzymes, including PTEN, therefore require reducing conditions for catalysis (Maehama & Dixon, 1998).

The findings of Leslie, et al (2003) indicated that as tumours, and some specialised immune cells such as phagocytes, produce high levels of ROS, this may represent a mechanism by which these cells down-regulate PTEN activity and thus evade apoptosis. Moreover, the ROS can be produced by the NADPH oxidase complex in the plasma membrane by binding PI3,4,5P3 (Welch, et al 2002). As PTEN's substrate PI3,4,5P3 is in close proximity to the ROS-producing complex, increases in PI3,4,5P3 via receptor signalling could be a mechanism of actually inhibiting PTEN.

1.2.3 PTEN & Attenuation of PI3K/Akt Signalling

Activation of the serine/threonine protein kinase Akt/PKB by PI3,4,5P3 is an important mechanism in cell survival, but one which must therefore be regulated. The observation that expression of PTEN caused an increase in levels of inactivated non-phosphorylated Akt indicated that PTEN prevents phosphorylation of Akt/PKB, thus antagonising PI3K activity. Conversely, deletion of PTEN results in constitutively activated, phosphorylated Akt (Cantley & Neel, 1999). By restraining Akt activation, PTEN could therefore prevent the activation of Akt substrates such as mTOR, PDE-3B and IKK, and promote activation of pro-apoptotic substrates such as BAD and FOXOs. The main physiological substrate of PTEN is the phosphatidylinositol lipid PI3,4,5P3, which is specifically dephosphorylated by PTEN at the 3' position on the inositol ring. The role of PTEN in the restriction of PI3K signalling and Akt/PKB activation is illustrated in Figure 4. Recently, an activation mechanism has been described for PI3,4,5P3 binding. In this model, PI4,5P2 binds to a distinct N-terminal site on PTEN and causes an allosteric conformational change in the active site which favours PI3,4,5P3 binding. A feedback loop therefore exists as PTEN regenerates its activator PI4,5P2 from PI3,4,5P3 (Campbell, et al 2003).

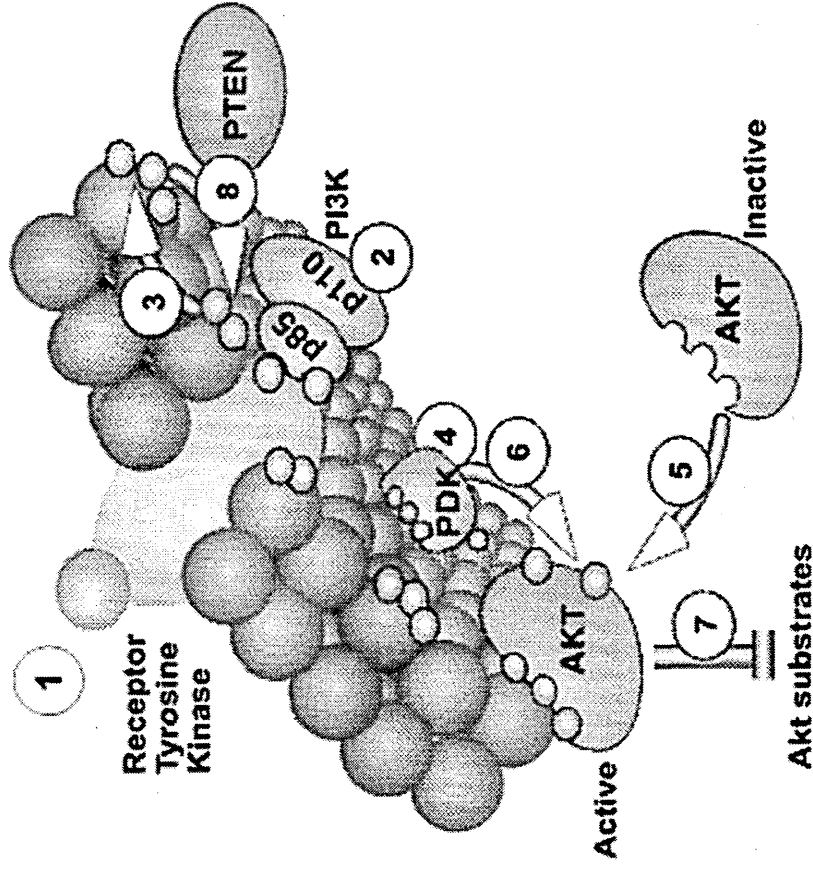


Figure 4 PTEN & the Akt/PKB Pathway

1. Growth factor binds to receptor and induces autophosphorylation, 2. PI3K is recruited to the receptor via p85 subunit, 3. Active PI3K phosphorylates PI4,5P2 (2 balls) to PI3,4,5P3 (3 balls), 4. PI3,4,5P3 binds PDK ser/thr kinases, 5. PI3,4,5P3 binds Akt ser/thr kinases, 6. PDK phosphorylates and activates Akt, 7. Activated Akt phosphorylates downstream targets, 8. PTEN antagonises the pathway by dephosphorylating PI3,4,5P3 to PI4,5P2. Adapted from Vazquez & Sellers, 2000.

Several components of the Akt/PKB pathway, including PTEN, are highly conserved in *C. elegans* (DAF-18) and *D. melanogaster* (dPTEN), underlining an evolutionarily important mechanism in development and homeostasis (Huang, et al 1999, & Gao, et al 2000). Work by Myers, et al (1998) and Furnari, et al (1999) revealed human mutations of PTEN in both germline and sporadic tumours which disrupted the lipid phosphatase activity. Additionally, PTEN-null cell lines (Myers, et al (1998) and murine PTEN^{-/-} fibroblasts (Stambolic, et al, 1998) showed increased levels of PI3,4,5P3. The PI3K inhibitor LY294002 can also mimic the effects of PTEN by effectively blocking Akt/PKB phosphorylation (Li & Sun, 1998).

Although PTEN reduces activation of Akt and therefore prevents cell cycle progression, the ultimate fate of the cell depends upon the cell type. PTEN can either cause G1 arrest or apoptosis via inhibition of the PI3K pathway, and evidence for these outcomes will be discussed.

1.2.3.1 PTEN & Induction of Cell Cycle Arrest

The mechanism by which PTEN can induce cell cycle arrest has been studied in a variety of cell types, but the consensus implicates inhibitors of cyclin-dependent kinases p27^{Kip1} as a target for PTEN. The induction of G1 cell cycle arrest in PTEN-null cell lines by addition of wild-type PTEN has been noted in the glioblastoma cell lines U87-MG and U178 (Li & Sun, 1998, Furnari, et al 1998 & Cheney, et al., 1998), 768-O renal carcinoma cells (Ramaswamy, et al 1999) MCF-7 (Weng, et al 1999) and ZR-75 (Hlobilkova, et al., 2000) breast cancer cell lines, several thyroid carcinomas (Weng, at al, 2001a), two bladder cancer cell lines (Tanaka, et al 2000) and several cell

lines of endometrial origin (Matsushima-Nishiu, et al 2001). This effect was only observed under low-serum conditions, suggesting that serum components such as mitogens can modulate the function of PTEN. Furthermore, addition of mutant forms of PTEN into the glioblastoma cell lines indicated that only the lipid phosphatase activity was essential for growth suppression by G1 arrest (Furnari, et al, 1998).

P27^{Kip1}

It has been shown by several groups that G1 growth arrest by PTEN is effected by up-regulation of p27^{Kip1} and down-regulation of cyclin D1 (Cheney, et al 1999, Medema, et al 2000, Weng, et al 2001b & Gottschalk, et al 2001), cyclin D3 (Zhu, et al 2001) or cyclins A, and B (Seminario, et al, 2003), and inhibition of Rb phosphorylation (Lu, et al, 1999, Paramio, et al 1999 & Radu, et al 2003). Notably, the expression of p27^{Kip1} is reduced in various tumour cell lines (Graff, et al 2000, Gesbert, et al 2000 & Yang, et al 2000).

By mutating key residues in the active site of PTEN, Weng, et al (2001b) determined that PTEN down-regulates cyclin D1 through its ambiguous protein phosphatase activity but up-regulates p27^{Kip1}, p57 and p21 (Wu & Li, 2000) via downstream events orchestrated through the lipid phosphatase activity. The p27^{Kip1}, p57 and p21 CDK inhibitors are therefore up-regulated via the inhibition of Akt/PKB by PTEN, possibly via activation and nuclear translocation of Forkhead transcription factors (FOXOs).

Nakamura, et al (2000) demonstrated that a constitutively expressed form of FKHR (FoxO1) which could not be phosphorylated by Akt/PKB could cause

G1 arrest or apoptosis in PTEN-null cells. PTEN also appears to be capable of affecting not only transcription of p27^{Kip1} but also the stability of the protein. Inhibition of Akt/PKB by PTEN causes a decrease in the levels of the ubiquitin E3 ligase SCF^{SKP2} which degrades p27^{KIP1}, thus contributing to p27^{KIP1} protein accumulation (Mamillapalli, et al 2001 & Nakayama, et al 2001). Interestingly, treatment of nasopharyngeal carcinoma cells with ceramide induced G1 arrest and up-regulation of p27^{Kip1} in a PTEN/PI3K independent manner, possibly via dephosphorylation of Akt by CAPP, a ceramide-activated protein phosphatase (Zhu, et al 2003).

GSK3 β and β -catenin

Other possible elements involved in the induction of G1 arrest by PTEN as a protein phosphatase are GSK3 β , involved in insulin-mediated signalling and the signalling/cell adhesion protein β -catenin (Polakis, 1998). β -catenin is involved in many pathways, with complex regulation, and when activated enters the nucleus to effect gene transcription (Stambolic, 2002). Two possible mechanisms of control by PTEN over the level of cyclin D1 have recently been proposed by Radu, et al, 2003.

The first is via phosphorylation of nuclear cyclin D1 by GSK3 β -mediated phosphorylation and inactivation of β -catenin, and the subsequent export of cyclin D1 to the cytoplasm for degradation. GSK3 β is activated when Akt/PKB is inhibited, thus PTEN may inhibit transcription and promote degradation of cyclin D1 by preventing nuclear accumulation of the protein. In support of this mechanism, levels of activated nuclear β -catenin have been shown to be reduced by exogenous PTEN expression (Persad, et al 2001).

Additionally, some tumours show activating mutations in the degradation targeting box of β -catenin, which is normally phosphorylated to cause nuclear exclusion and breakdown of the protein (Koch, et al 1999, Fukuchi, et al 1998 & Palacios & Gamello, 1998).

The second mechanism proposes that PTEN decreases cytoplasmic 'free' cyclin D1 by a GSK3 β -independent pathway, which has been reported previously (Germain, et al (2000), although the exact mechanism of degradation is unknown. The novel tumour suppressor MDA-7 has recently been shown to down-regulate PI3K, redistribute β -catenin to the cytoplasm and up-regulate GSK3 β and PTEN levels in breast and tumour cells, adding another regulatory mechanism to these pathways (Mhashilkar, et al 2003).

1.2.3.2 PTEN & Induction of Apoptosis

Reconstitution of wild-type PTEN into some PTEN-deficient cell lines may induce apoptosis rather than cell cycle arrest. Restoration of wild-type PTEN to several breast cancer cell lines induced G1 arrest followed by apoptosis (Li, et al., 1998) and similar results have been observed in endometrial cell lines (Sakurada, et al., 1999). Apoptosis was also induced by PTEN restoration into glioma cell lines (Davies, et al, 1998, Wick, et al 1999 & Tian, et al.,1999). Under serum-starved conditions apoptosis is induced rapidly in breast cancer cells (Lu, et al, 1999) and more slowly in prostate carcinoma cells (Davies, et al, 1999). One explanation is that under conditions where apoptotic stimuli are absent (eg Fas ligand, TNF) PTEN causes early G1 arrest. PTEN could then inhibit survival by growth factors and induce

apoptosis, depending upon the strength of the proliferative extracellular signal (Di Christofano & Pandolfi, 2000).

clAP (Inhibitor of Apoptosis) Proteins

It has been shown that PTEN reconstitution into PTEN-null endometrial cell lines Ishikawa and RL-95-2 causes a decrease in expression of the survival factor clAP-1 (Gagnon, et al, 2003). Human inhibitors of apoptosis, (IAP), proteins belong to a family consisting of six members which can inhibit the proteolytic activity of caspase-3, -6 and -7. Downstream activation of pro-caspase 9 is therefore prevented, and the cell escapes apoptosis induced by pro-apoptotic stimuli such as Fas, chemotherapeutic agents and serum starvation (Wang, et al 2003).

NF κ B

PTEN may also initiate apoptosis via the inhibition of NF κ B induced by TNF- α . This has been shown to occur via two possible mechanisms. Firstly, by inhibition of Akt/PBK which can activate IKK α and NF κ B, in a manner that appears to be cell-type specific (Gustin, et al 2001). Secondly, PTEN has been shown to inhibit NF κ B independently of I κ B degradation (Koul, et al 2001), possibly by preventing TNF-mediated activation of the p65 transactivation domain of NF κ B, which is essential for activating the NF κ B complex (Mayo, et al 2002). The exact mechanisms are yet to be determined, and may involve complex feedback regulation of PTEN itself by the NF κ B pathway (Kim, et al, 2003).

1.2.4 Expanding the Cellular Role of PTEN

PTEN has become established as a key player in the regulation of phospholipid signalling through Akt/PKB, but a definitive *in vivo* substrate for its *in vitro* protein phosphatase activity remains elusive. Additionally, other downstream effects of PTEN are becoming apparent, suggesting that the tumour suppressor could be involved in multiple signalling pathways involving both protein and lipid phosphatase functions.

1.2.4.1 PTEN & Cell Migration

As PTEN is a protein phosphatase *in vitro* against acidic phospho-Tyr/Ser/Thr peptides, it has been postulated that the integrin-mediated signalling proteins FAK (focal adhesion kinase) and Shc may be targets. These proteins are involved in cell motility and spreading, and modulation by PTEN could contribute to growth suppression. Loss of PTEN is correlated with anchorage-independence, and in PTEN-null tumour cells expressing exogenous PTEN, forced detachment from the ECM causes up-regulation of PTEN (Wu, et al 2002). PTEN does exhibit dephosphorylation of FAK and Shc *in vitro*, and work by Gu, et al (1998) & Tamura, et al (1998 & 1999) proposed this to be the case *in vivo* using U87-MG cells.

However, lipid phosphatase mutants of PTEN with intact protein phosphatase activity have been shown to have no effects on FAK phosphorylation or motility (Maier, et al, 1999 & Liliental, et al, 2000), and other groups have been unable to repeat the original work in U87-MG. Additionally, evidence may link PTEN directly to FAK and Shc through its normal lipid phosphatase role in the Akt/PBK pathway (Lopez-Illascale, et al, 1997 & Casamassima &

Rozengurt, 1998), thus implicating PTEN in MAPK pathway responses. Work in human PC3 cells implicates the integrin-linked kinase (ILK) in attachment. Here, ILK is bound and activated by the ILK-activating proteins CH-ILKBP and localised to focal adhesions, where Akt/PKB is stimulated and GSK3 β is inhibited. The generation of PI3,4,5P3 further activates ILK, causing re-organisation of F-actin and paxillin to direct attachment and migration. PTEN appears to disrupt the ILK:CH-ILKBP complex by an unknown mechanism (Attwell, et al 2003).

It now appears that mechanisms exist to compartmentalise PTEN during chemotaxis and prevent it from disrupting complexes forming at the leading edge of the motile cell. Recent studies in mammalian leukocytes and the amoebae *D. discoideum* have shown that when sensing cAMP as a chemoattractant, PI3K localises to the leading edge of the cell where a PI3,4,5P3 gradient forms. This enhances signalling through Akt/PKB and directional movement, whilst PTEN is localised to the posterior away from the concentrated PI3,4,5P3.

Cells lacking PTEN show multiple, random pseudopodia and a lack of directional movement (Comer & Parent, 2002 & Iijima & Devreotes, 2002). The localisation mechanisms of PI3K and PTEN are currently under investigation. In *D. discoideum*, recent work suggests that activation of chemoattractant receptors provides G-protein-coupled binding sites on the cytosolic face of the plasma membrane which bind PI3Ks at the leading edge (Huang, et al 2003).

A study of chemotaxis in neutrophils concurred with the findings in *D. discoideum*, and described a mechanism for localising PTEN via the

cytoskeletal proteins PIX α (PAK-associated guanine nucleotide exchange factor) and Cdc42. In this system, the two proteins direct F-actin formation at the leading edge, but PIX α actively repels PTEN (Zhong, et al 2003).

1.2.4.2 PTEN and Regulation of Pathways Other Than PI-3 kinase

MAPK Signalling

The MAP and ERK kinase pathways are activated in response to binding of cell surface receptors such as EGF, or the interaction of integrins with the extracellular matrix to control adhesion, growth and differentiation. The pathways are redundant and partake in complex cross-talk; a diagrammatic overview of MAPK/ERK is shown in Figure 5. The Shc adaptor couples receptor tyrosine kinases and associated receptors to these pathways, and is believed to be regulated by recruitment from the cytosol to the plasma membrane. Here, it binds Grb-2/Sos complexes and causes transfer of the guanine nucleotide exchange factor Sos to Ras, resulting in Ras activation (Plyte, et al 2000). Downstream of Ras are members of the Raf family which activate MAPKs/ERKs and/or JNK/SAPK to effect gene transcription.

PTEN has been shown to be involved in the MAP/ERK kinase pathway in several ways. Firstly, PTEN can act in a Shc-dependant manner, dephosphorylating this protein and inhibiting the formation of Grb-2/Sos complexes and thus activation of Ras (Gu, et al 1998). Secondly, PTEN can inhibit MAPK and ERK via Shc-independent mechanisms involving Gab1 and IRS. Gab1 contains a PH domain that interacts with PI3,4,5P3-rich membranes (Ong, et al 2001) via PI3K and the tyrosine phosphatase SHP2.

MAPK/ERK in Growth and Differentiation

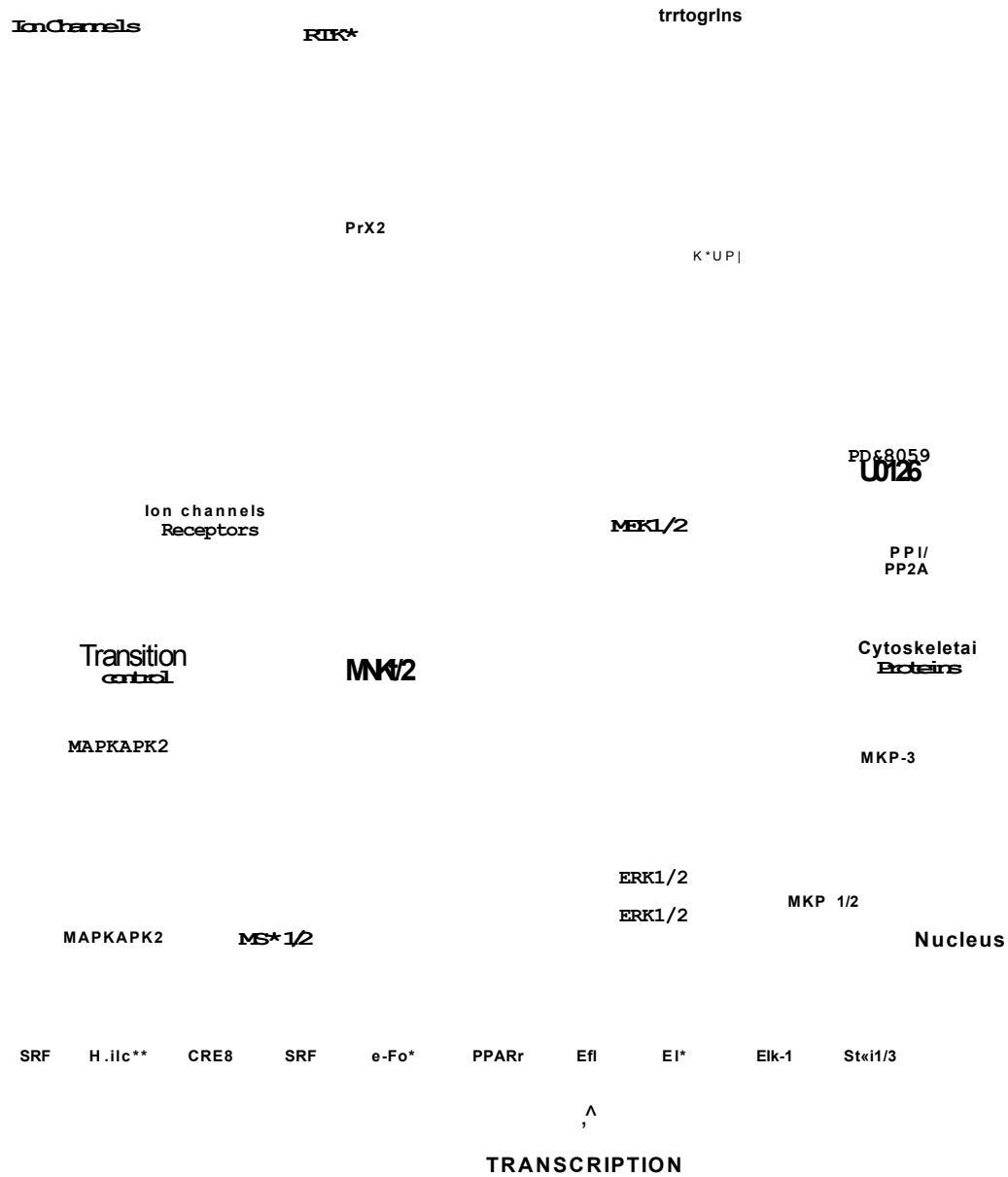


Figure 5 MAPK/ERK Pathways Taken from www.cellsignal.com

Dephosphorylation of PI3,4,5P3 could prevent Gab1 from interacting with SHP2, thus inhibiting MAPK activation (Yart, et al 2001). Recently, Gab1 decoys have been shown to inhibit EGF-induced ERK and Akt/PKB activation in tumour cell lines lacking functional PTEN (Ren & Wu, et al 2003).

Several groups have now reported that PTEN negatively regulates ERK-mediated signalling of estradiol in endometrial carcinoma (Zhang, et al 2003) and hepatoma cell lines (Marino, et al 2003), and that ERK may regulate PTEN levels. Binding of PDGF to its receptor activates both PI3-K and ERK/MAPK pathways. Work by Mahimainathan & Ghosh (2004) has demonstrated that PTEN binds directly to the cytosolic domain of the PDGF receptor through its C2 and C-terminal domains, causing dephosphorylation of the autophosphorylated tyrosine residues in the receptor. The group propose that PTEN may inactivate other receptors, such as for EGF, in this manner to regulate signalling in the absence of ligand binding.

A role for PTEN in negatively regulating BCR (B-cell antigen receptor)-mediated proliferation has recently been described (Brown, et al 2004). The low-affinity IgG receptor FcγRIIb, involved in immune regulation, is thought to recruit PTEN and the immune cell-restricted 5' inositol phosphatase SHIP (Kalesnikoff, et al 2003). PTEN converts PI3,4,5P3 to PI,4,5P2, whilst SHIP uncouples BCR from the GTPase Ras and converts PI3,4,5P3 to PI3,4P2, the overall effect being to prevent ERK/MAPK activation.

The involvement of PTEN in insulin signalling is well known, and it is believed that it may achieve attenuation of the pathway via direct dephosphorylation of IRS-1. This would prevent the formation of an IRS-1/Grb2/Sos complex and thus MAPK activation (Weng, et al 2001c & Yeon, et al 2003). Up-regulation

by PTEN of IRS-2, which is a component of insulin signalling, has also been observed (Simpson, et al, 2001). Work by Weng, et al (2003) has demonstrated that in MCF-7 breast cancer cells, PTEN can inhibit insulin-mediated signalling, but not EGF-mediated signalling, by blocking phosphorylation of the transcription factor ETS-2. This was shown to occur independently of PI3K via ERK/MAP kinases, and involved the protein phosphatase activity of PTEN. The apparent specificity of PTEN as a protein phosphatase for different receptor tyrosine kinases implies that the role of PTEN may be cell-type specific, and that therapeutic targeting of PI3K/Akt in some cancers may not be effective.

Hypoxia & Angiogenesis

Under 'normoxic' conditions, PTEN has been shown to downregulate hypoxia-inducible factor 1 α (HIF-1 α), normally expressed under low oxygen conditions or by EGF stimulation (Zhong, et al 2000 & Zundel, et al 2000). HIF-1 α is a transcriptional activator of genes for glucose transporters, glycolytic enzymes and VEGF, the latter of which is commonly up-regulated in tumours and increases vascularisation. Recently, restoration of PTEN into glioma cells has shown that PTEN down-regulates HIF-1 α and VEGF expression, with an increase in p27^{Kip1} orchestrated via PI3K and not p38/MAPK (Gomez-Manzano, et al 2003 & Abe, et al 2003). Similar findings have been made in gastric carcinomas, where a lack of PTEN causes increased MMP-7 expression, (Zheng, et al 2003) which could synergise with VEGF to contribute to increased angiogenesis and invasiveness. Conversely, over-expression of wild-type PTEN in primary microvascular

endothelial cells stimulated with VEGF caused inhibition of angiogenesis (Cai, et al 2003). Interestingly, in cells lines with abnormally expressed pro-apoptotic Myc, which cannot activate p53 death genes (Bringold & Serrano, 2000), PTEN and HIF transcription were unaffected and hypoxia-induced death occurred by other mechanisms (Brunelle, et al 2003). It is now known that a close relationship exists between PTEN and p53, which will be discussed in Section 1.3.

Androgen Insensitivity

Androgen insensitivity is a feature of some prostate cancers, and it has been demonstrated that androgens can protect prostate cancer cells from PTEN-induced apoptosis via Akt/PKB (Li et al 2001). This is probably due to aberrant ligand binding and activation of the androgen receptor (AR), or activation via MAPK pathways (Ueda, et al 2002) which could explain why the AR is required for survival. Unsurprisingly, loss of functional PTEN could enhance AR signalling and progression to the androgen independent state (Nan, et al 2003). The exact mechanisms involved are controversial, but evidence exists for two Akt consensus sites, Ser213 and 791, in AR which Akt can phosphorylate to activate the receptor (Lin, et al 2001).

Antagonism of PI3K by PTEN therefore leads to negative regulation of AR signalling in an Akt-dependant manner (Sharma, et al 2002), and down-regulation of the AR co-activator β -catenin has also been observed in this model (Trucia, et al 2001). Another group have recently detected binding of FKHR/FOXO1 to the NT region of AR which prevents transcription of pro-

apoptotic genes. As Akt/PKB can also inhibit FOXOs, this may represent multiple mechanisms by which prostate cancers survive (Li, et al 2003).

Inositol Phosphates and PTEN Substrate Specificity

It is accepted that inositol lipids and inositol phosphates have their own dedicated phosphatases and kinases, with little cross-over in substrate specificity. Until recently, PTEN was thought to exhibit negligible activity towards inositol phosphates and has been shown to be a poor 3' phosphatase towards inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄) (Maehama & Dixon, 1999). Evidence was presented for the hydrolysis of inositol 1,3,4,5,6-pentakisphosphate (InsP₅) by PTEN *in vitro* via its 3'-phosphatase activity to Ins(1,4,5,6)P₄ (Caffrey, et al 2001). Ins(1,4,5,6)P₄ is an antagonist of PI3,4,5P₃ (Eckmann, et al 1997) and these substrates could theoretically compete for PTEN binding. Ins(1,4,5,6)P₄ is also associated with transcriptional regulation (Odom, et al 2000), and Rho-GTPase activation (Zhou, D et al, 2001).

Work by Orchiston, et al (2003) suggest that wild-type PTEN, which is active against 3' phosphatidylinositol phospholipids, does not regulate inositol phospholipids *in vivo*. This group used a C2 domain mutant form of PTEN (M-CBR3) initially described by Lee, et al (1999). The mutant had little effect on 3'-phosphoinositide lipids, but selectively dephosphorylated InsP₅. Decreased levels of InsP₅ corresponded with a decrease in cell growth and anchorage-independence, suggesting that InsP₅ acts as a proliferative agent of unknown physiological role.

1.3 Regulation of PTEN Expression

Despite the large amount of data regarding the role of PTEN in the control of various important pathways, detail regarding the regulation of mRNA and protein levels has only recently begun to emerge. Several mechanisms have been proposed for the regulation of PTEN expression, including transcriptional control via interaction with p53, PPAR γ and Egr-1, promoter methylation (see Section 1.5) and modulation of protein expression by cytokines.

1.3.1 Transcriptional Control of PTEN mRNA

Interaction of the PTEN Promoter with p53

The tumour suppressor p53 is well-characterised and one of the most commonly mutated genes in cancer. The p53 protein acts as a transcription factor to induce growth arrest or apoptosis by up-regulating pro-apoptotic genes such as p21, and Bax. Activity of the p53 protein is regulated by a feedback loop with MDM2, which p53 up-regulates. MDM2 protein binds to p53, inhibits its transcriptional activity and causes nuclear exclusion and proteasome degradation (Freedman & Levine 1998). p53 can be inactivated by growth factors and hormones such as oestrogen, and expression of such stimulatory factors is often uncontrolled in tumours, as is the case in breast carcinomas (Molinari, et al 2000).

The discovery of a putative p53 binding site in the promoter of PTEN between -1190 and -1157 (Stambolic, et al 2001) indicated a possible transcriptional mechanism for PTEN regulation (see Figure 2). This group reported transactivation of PTEN by p53 via transfection into the PTEN-null

U87-MG glioblastoma cell line, and defined the region between -1001 and -427 as a positive regulatory element which drives constitutive PTEN expression. Both mRNA and protein levels of PTEN were significantly increased. Analysis of p53-mediated apoptosis revealed that the p53 binding sites in the PTEN promoter were essential for efficient p53-mediated cell death, and a lack of functional PTEN inhibited this process (Mayo & Donner, 2002).

Recent evidence of decreased PTEN expression in p53^{-/-} mice supports the idea that p53 up-regulates PTEN (Hesselager, et al 2003) and that p53 and PTEN proteins can physically bind *in vivo* and *in vitro* to regulate PI3K signalling and also to protect p53 from MDM2-mediated degradation (Zhou, et al 2003). Moreover, the PI3K inhibitor LY294002 was shown to synergise with introduction of exogenous PTEN into U87-MG cells to transactivate p53 expression and its target genes p21^{Waf-1} and IGFBP3 (Su, et al 2003).

The requirement of both PTEN and p53 for effective suppression of tumours has been reinforced by work using irradiated mice. Here, PTEN heterozygotes were just as sensitive to irradiation and lymphoma development as p53 heterozygotes, suggesting that the two genes are complementary (Mao, et al 2003).

The hepatitis B virus X protein (HBx), which is involved in hepatocellular carcinoma, has been shown to interfere with p53 binding to its binding site on the PTEN promoter and thus down-regulates PTEN transcription (Chung, et al 2003). Hepatitis B infection may therefore promote tumourigenesis via inhibition of PTEN through decreased p53 transactivation, and the resulting downstream activation of Akt/PKB.

PPAR γ

The nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR γ) is a transcription co-factor that has been shown to regulate differentiation and/or cell growth in a number of cell types (Chen, et al 2003a). It acts as an anti-inflammatory mediator and may also act as a tumour suppressor, and is a therapeutic target for type-II diabetes. Patel, et al (2001) demonstrated that the PPAR γ ligand rosiglitazone (an insulin-sensitising drug) upregulates PTEN expression in human macrophages, MCF7 breast cancer cells and Caco2 colorectal cancer cells, and decreases Akt/PKB phosphorylation. Two PPAR γ binding elements were identified in the PTEN promoter at positions -15376 and -13339bp upstream of the PTEN transcription start site (assigned at -1035 relative to the translational start site).

Other groups have shown PPAR γ -mediated up-regulation of PTEN in pancreatic tumour cell lines (Farrow & Evers, 2003), and vascular endothelial cells (Goetze, et al 2002). These results suggest that PTEN up-regulation may be one mechanism by which PPAR γ agonists ameliorate inflammation and tumourigenic processes. One problem with this is that PPAR γ agonists, such as rosiglitazone, are used successfully to treat type II diabetes (Poulsen, et al 2003), which logically could cause potentially harmful elevation of PTEN. However, as PTEN also up-regulates IRS-2 which leads to an insulin-mediated increase in Akt/PKB activity (Simpson, et al 2001), PPAR γ agonists would be beneficial in this disease.

Egr-1

The immediate-early *Egr-1* is a transcription factor that is known to respond to the breast cancer mitogen insulin-like growth factor II (IGF-II) via IRS-1 (Tsuruzoe, et al 2001), and up-regulates the IGF-II promoter in response to hypoxia (Bae, et al 1999). Work by Virolle, et al (2001) identified a functional *Egr-1*-binding site (GCGGCGGCG) in the PTEN 5' untranslated region between positions -947 and -939, which up-regulated PTEN transcription in response to irradiation in mice. Moorehead, et al (2003) demonstrated induction of PTEN transcription via *Egr-1* in developing mammary cells stimulated with IGF-II, but not in mammary cells undergoing involution (Moorehead, et al (2001). The differences in response seen between developing and involuting mammary cells may reflect the divergent hormonal exposure of the cells. Moorehead, et al (2003) also proposes that the induction of PTEN could involve a regulatory loop where IGF-II signals through *Egr-1* to induce PTEN, and then *Egr-1* also induces IGF-II.

TGF-β1

Transforming growth factor-β1 is a pleiotropic cytokine which is capable of exerting a huge range of cellular effects, including proliferation, differentiation and apoptosis. Several papers have reported modulation of expression of PTEN by TGF-β1, but the data is conflicting. Li & Sun, (1997) first described rapid down-regulation of PTEN (TEP1) mRNA by TGF-β1 in a responsive asynchronously growing HaCaT keratinocyte cell line. A second group have also demonstrated PTEN mRNA down-regulation in pancreatic adenocarcinomas over-expressing TGF-β1 and synchronous (serum-starved)

cell lines treated with TGF- β 1 (Ebert, et al 2002). These two reports concluded that a reduction in PTEN may impart a growth advantage under TGF- β 1 stimulation, and may contribute to aggressiveness in the pancreatic study.

A third report, however, described the up-regulation of PTEN mRNA in a synchronous U937 monoclastic leukaemia cell line in response to TGF- β 1 (Lee, et al 1999b). The study also found up-regulation of anti-apoptotic Bcl-XL and resistance to Fas-mediated apoptosis. These apparently contradictory findings could be due to various factors, including cell type, dose of cytokine, and incubation times and conditions of growth. In light of current understanding, the results presented by (Lee, et al 1999) may be explained by the inhibition of PTEN protein by one or more of the various mechanisms previously described.

The TGF- β superfamily contains over 30 members with three isoforms, β 1, 2 and 3, but only TGF- β 1 will be discussed here. TGF β isoforms signal through ser/thr kinase receptors type I and II (T β RI and II). Binding of TGF to the constitutively active T β RII initiates recruitment of the receptor/ligand to T β RI and transphosphorylation of T β RI at its GS-box (juxtamembrane gly/ser-rich region). This creates a binding site for Smads, the signal transducing elements of TGF β which are activated by phosphorylation of their C-termini, and the internalisation of the receptor into endosomes for recycling.

Eight vertebrate Smads have been identified. These include receptor-activated Smads (R-Smads) Smad2 and 3 for the TGF-beta/activin pathway, or Smad1/5/8 for the bone morphogenetic pathway, (BMP). C-terminal phosphorylation of Smads by activated receptors results in their binding to

the common signalling transducer (Co-Smad) Smad4 and translocation to the nucleus. The R-Smads also compete for binding to T β RI with inhibitory Smads (I-Smads) Smad 6 and 7 (Attisano & Lee-Hoeflich, 2001).

The Smads have a conserved, modular structure composed of MH1, linker and MH2 domains. The MH1 domain contains a DNA-binding motif, an NLS and regions for binding transcription factors such as ATF-2, c-Jun/Fos, Forkheads and Sp1 (Shi, et al 2003). The linker contains consensus sites for MAPK phosphorylation, which causes sequestration of R-Smads to the cytoplasm and prevents nuclear translocation, for example the R-Smad1 is phosphorylated by ERK (Kretzschmar, et al 1999). In both R- and I-Smads this region also contains sites recognised by E3 ubiquitin ligases Smurf1 and 2 for degradation, and in the Co-Smad4 the linker has an additional nuclear export signal (NES).

The MH2 domain mediates many protein-protein interactions including Smad-receptor association and the formation of Smad homo- and heteromeric complexes consisting of up to six units. For example, Smads 2,3, and 4 are required as a complex to effect transcription of the CDK p15^{INK4B} via SP1 transcription factor (Li, et al 1995 & Feng, et al (2000). These complexes are thought to permit differential signalling thresholds to act on target gene expression.

Phosphorylated Smads are released from microtubules (Dong, et al 2000), and inhibitory I-Smads, and bind to endosomes containing transport proteins (Di Guglielmo, et al 2003), all of which increase Smad activation (Lutz and Knaus, 2002). Association with Smad4 and other R-Smads follows, and the complexes translocate to the nucleus via their NLS. Here, many genes are

activated as previously mentioned, but others are repressed to inhibit cell cycle progression, such as c-myc (Chen, et al (2002). An overview of TGF- β 1 signalling through Smads is shown in Figure 6.

TGF-β Signaling Pathway

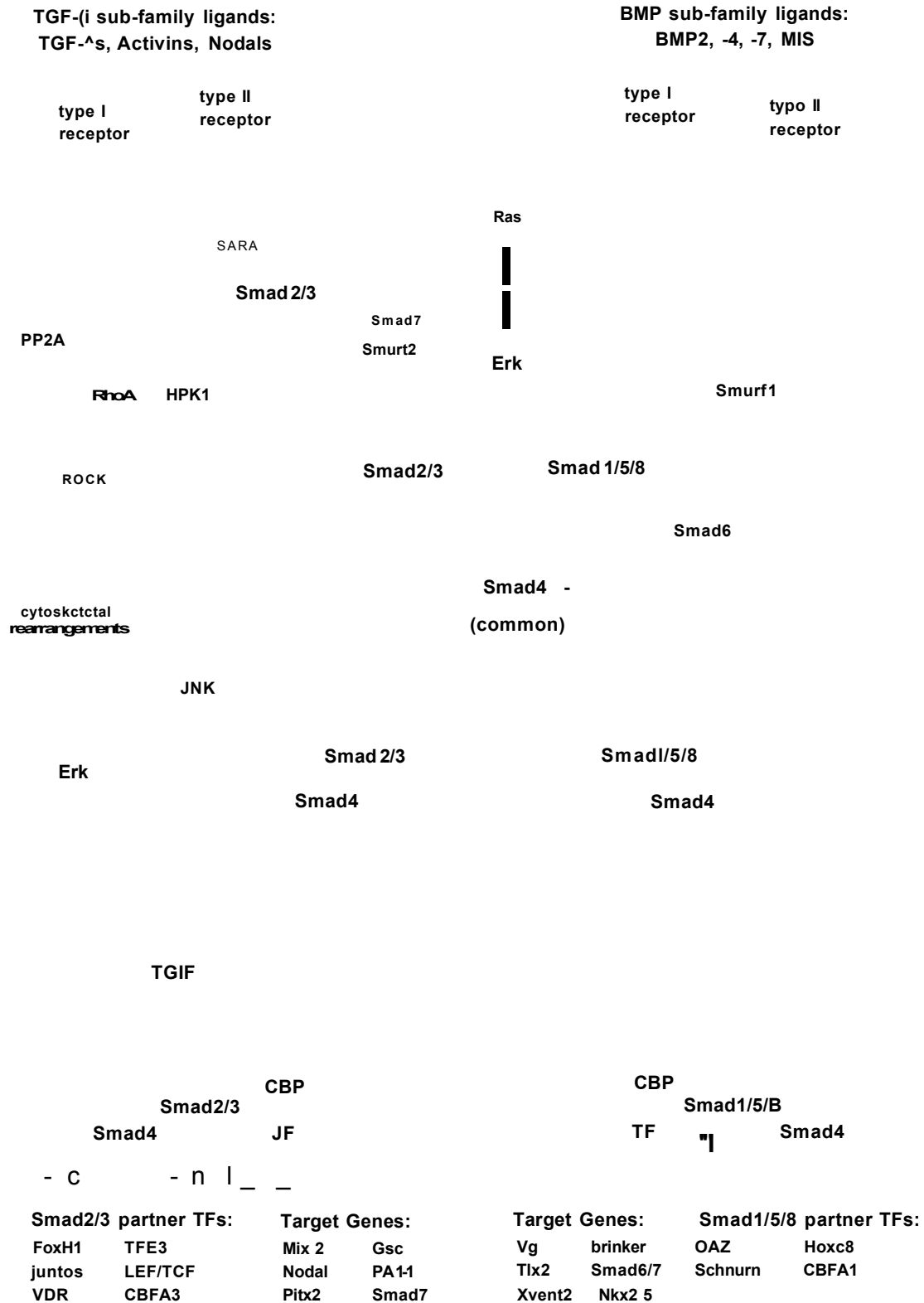


Figure 6 TGF-β Signalling to the Nucleus

Figure legends are overleaf. Taken from www.cellsignal.com

In addition to the traditional Smad-dependant signalling, TGF- β 1 also activates p38 MAPK/ERK/JNK pathways which are able to regulate Smads themselves. However, the activation-response kinetics are too rapid to be Smad-dependent, suggesting an alternative mechanism (Massague, 2000). Activation of Ras by TGF- β 1 has been observed in induction of ERK/MAPK (Yue, & Mulder, 2001), and the TGF β -activated kinase-1 (TAK1) is a MAPKKK that can activate both p38MAPK and JNK (Dowdy, et al 2003). TAK1 can also activate anti-apoptotic NF- κ B by phosphorylating I κ B, thus TGF- β 1 may also be involved in NF- κ B-mediate responses (Bhat, et al 2003). Work by Vasudevan, et al (2004) has demonstrated that NF- κ B is capable of inhibiting PTEN transcription through its p65 subunit by sequestering the transcriptional co-activators CBP/p300. Thus, during pro-apoptotic TNF stimulus, NF- κ B can prevent apoptosis by down-regulating mRNA and protein levels of PTEN.

Other pathways may be activated in a cell-type specific manner. These include Cdc42 and Rac, also involved in MAPK and cytoskeletal arrangement, and Rho-like small GTPases which may effect gene expression responses (Derynck & Zhang, 2003). Additionally, TGF- β 1 can down-regulate Rb and a variety of associated CDKs and cyclins to cause G1 arrest (Hu, et al 2001 & Bhowmick, et al 2003).

TGF- β 1 has also been widely implicated in cancer, but its involvement is complex. During the early stages of tumourigenesis, the cytokine acts to suppress tumour formation via its cell-cycle restrictive functions detailed above. However, during neoplastic change, many tumours show progressive lack of responsiveness to growth inhibition by TGF- β 1 and actually produce

large amounts of the protein (Boyd & Kaufman, 1990 & Lei, et al 2002). This seems to provide autocrine stimulus to the cells which increases angiogenesis and motility, via up-regulation of plasminogen activator inhibitor-1 (PAI-1) (Kutz, et al (2001) and has an immunosuppressive effect (Reiss, 1999).

Tumours may display mutant TGF receptors which signal in an aberrant manner, most commonly in T β RII, (Oft, et al 1998, Heldin, et al 1999 & Waite & Eng, 2003) and/or Smads, have reduced levels of the CDK inhibitors such as p27^{Kip1}, increased MDM2 which inactivates p53 (Gold, 1999) and increased cyclin D1 (Kornmann, et al 1999). Steroid hormones such as oestrogen are also implicated in abnormal TGF- β 1 signalling, and have been shown to down-regulate T β RII and allow ovarian cancer cells to evade TGF- β 1-mediated growth suppression (Evangelou, et al, 2000 & Frasor, et al 2003).

Of particular relevance to this thesis, TGF- β 1 has been shown to activate PI3K through Smad2 (Bakin, et al 2000), ERK/MAPK (Lhuillier & Dryer, 2003) and may invoke EGF receptor activation, (Vinals & Pouyssegur 2001).

1.3.2 Post-Translational Modulation of PTEN Protein Expression

Progesterone & oestrogen

The high incidence of PTEN loss or mutation in some cancers, and in particular endometrial carcinomas (see Section 1.5), has indicated that steroid hormones could play a role in PTEN regulation. Studies on PTEN expression in the endometrium, which undergoes complex morphological changes in response to cyclic levels of oestrogen and progesterone, have

shown that these hormones do indeed regulate PTEN mRNA and protein expression levels.

Mutter, et al (2000) demonstrated that levels of PTEN mRNA in endometrial cells were higher during the post-ovulatory secretory phase compared to the proliferative phase of the menstrual cycle. The secretory phase has a high progesterone level whilst the proliferative phase is dominated by oestrogens, suggesting that progesterone is responsible for changes in PTEN levels. The protein levels and localisation were more complex and cell-type specific. Oestrogen-driven dividing epithelial and stromal cells were seen to have nuclear and cytoplasmic protein, with increased nuclear localisation during decidualisation and menstrual apoptosis. During the secretory phase, progesterone levels rise which caused epithelial cells to lose PTEN protein whereas stromal cell levels increased, particularly in the cytoplasm.

An investigation into PTEN expression in the endometrium was made in the light of the current evidence for PTEN protein regulatory mechanisms. Guzeloglu-Kayisli, et al (2003) have described PTEN expression in the menstrual cycle and early pregnancy consistent with the work of Mutter, et al (2000). This group showed that estradiol increased phospho-PTEN after 5-15 minutes of treatment in stromal cells, which could account for the observation that during the proliferative phase these cells are resistant to apoptosis. Additionally, the oestrogen receptor- α is activated by CK2 which inactivates PTEN, providing an additional feedback mechanism for increased oestrogen signalling and PTEN down-regulation. Progesterone increased PTEN protein levels after 24 hours in stromal and glandular cells, and in decidual and glandular cells during pregnancy.

This suggests that this hormone may dephosphorylate PTEN and increase protein activity levels in a long-term manner, to cause the increase in apoptosis observed in these cells. In conclusion, Guzeloglu-Kayisli, et al 2003 proposed that the two steroid hormones may target PTEN to affect the concentration and spatial localisation of the protein in different endometrial compartments.

Other Positive Modulators of PTEN Protein Expression

Several additional factors have also demonstrated the ability to up-regulate PTEN expression, these being a vitamin D3 analogue, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and bone morphogenetic protein (BMP).

Vitamin D3 Analogues

Vitamin D3 is a secosteroidal hormone involved in bone metabolism, which is also capable of inhibiting proliferation and inducing differentiation in tumours of diverse origin (Banerjee & Chatterjee, 2003). The growth inhibition effects of this molecule are thought to be via G₀/G₁ arrest and up-regulation of CDK inhibitors such as p27^{KIP1}, p21^{Waf1} and induction of p53 and apoptosis (Yang & Burnstein, 2003, Audo, et al 2003 & Flanagan, et al 2003). Despite its efficacy against tumours, it is of little clinical use as at the doses required for effectiveness a side effect of treatment is calceamia (Kensler, et al 2000). Novel D3 analogues have been designed to be anti-proliferative without the dangerous side effects (Chen, et al 2003b).

The novel D3 analogue Gemini-19-nor was shown to be more potent in mediating growth, G₀/G₁ arrest and p27^{KIP1} expression, apoptosis and differentiation than vitamin D3 in leukaemic, breast and prostate cancer cell lines. Interestingly HL-60 myeloid leukaemic cells, which are wild-type for PTEN, also demonstrated up-regulation of PTEN protein during Gemini-19-nor -induced differentiation (Hisatake, et al 2001). This group also showed up-regulation of PTEN in HL-60 cells treated with the phorbol diester TPA and all-*trans*-retinoic acid (ATRA), which induced macrophage-like or granulocyte-like differentiation respectively in this cell line.

These results suggest that PTEN is involved in myeloid differentiation, and may indicate that vitamin D3 analogues are a potential therapy for myeloid leukaemias. Another D3 analogue, Seocalcitol (EB1089), has been shown to inhibit the growth of aggressive pituitary tumours without increasing PTEN expression (Liu, et al 2002), highlighting the cell-type specificity of PTEN involvement.

NGF & BDNF

It has been reported that NGF, which stimulates neurite extension, upregulates PTEN mRNA levels in rat neuronal pheochromocytoma PC12 cells (Li & Sun, 1997). An increase in PTEN protein has also been observed in the same cell line stimulated with NGF (Lachyankar, et al 2000). This group also demonstrated increased PTEN protein in immature and mature mouse CNS stem cells during BDNF-induced differentiation. PTEN protein could only be detected weakly in immature astrocytic cells and was absent in mature astrocytes. This indicates that PTEN plays a role in neural

development and the extension of neurites, and that perhaps the absence of PTEN in glial precursors (astrocytes) is important for glioma development.

Bone morphogenetic protein (BMP).

BMP signals through the TGF β -family receptor BMPR1A, and mutations in this receptor have been noted in patients with Cowden disease, a proliferative disorder associated with PTEN mutation (see Section 1.4). Recently, Waite & Eng, (2003b) demonstrated up-regulation of PTEN protein in MCF-7 breast cancer cells stimulated with BMP2. The increase in PTEN protein was due to inhibition of degradation, through inhibition of association of PTEN with ubiquitin-conjugating enzymes UbCH7 and UbC9.

1.4 PTEN and Disease

It is now apparent that mutation or loss of PTEN is a feature of many malignant and non-malignant human proliferative disorders, ranging from aggressive carcinomas to benign hamartomas. Aberrations in genomic PTEN contribute to tumourigenic phenotypes in a cell-type specific manner and may be germ-line or somatic. The incidence of PTEN mutation or loss is strikingly high for some cancers, notably endometrial, but almost absent from haematological malignancies. The current understanding of the contribution of PTEN mutation or loss in human proliferative diseases is discussed below.

1.4.1 Germline Mutations

PTEN Hamartoma-Tumour Syndrome (PHTS)

PHTS broadly identifies several related autosomal dominant disorders

resulting from germline PTEN mutations, which display overlapping phenotypes. Diagnosis can be problematic as the disorders can share both phenotypic features and identical PTEN mutations. It is likely that individual genotypes, with loss or mutation of other disease-related genes, contribute to this lack of distinction, as a small number of patients do not have PTEN mutations (Merks, et al 2003 & Waite & Eng, 2002). The four main disorders, Cowden disease (CD), Bannayan-Ruvalcaba-Riley syndrome (BRRS), Lhermitte-Duclos disease (LDD), and Proteus syndrome (PS) are described below.

Cowden Disease (CD)

CD is associated with a susceptibility to breast and thyroid cancers, increasing the risk of breast cancer in affected females to 25-50% and lowering the age of tumour development by approximately 10 years from the population average. Affected individuals commonly display hamartomas, or mucocutaneous lesions (papillomas and trichilemmomas), carcinomas of breast, thyroid follicular carcinoma and macrocephaly.

Approximately 10-50% of CD cases are familial, but the true figures are unknown due to underdiagnosis. Up to 80% of CD cases contain a germline mutation of PTEN, and mutation of PTEN correlates with tumour aggressiveness (Zhou, et al 2003b & Marsh, et al 1998). Many such mutations in the PTEN gene have been described, and ~65% of these appear to affect the first 5 exons coding for the phosphatase domain and the promoter region (Eng, 2003).

In a murine model developed by Podyspanina, et al (1999), mice containing a deletion in exon 5 developed neoplasms in multiple organs reminiscent of CD and BRRS . Cases of CS without intragenic PTEN mutation could be due to mutations in BMPR1A (Waite & Eng 2003b), or promoter mutation which has been detected in ~10% of samples (Zhou, et al 2003b).

Bannayan-Ruvalcaba-Riley syndrome (BRRS)

This familial disorder is characterised by lipomatosis, speckled penis, hemangiomas and macrocephaly, and ~60% of patients harbour a germline PTEN mutation (Hendriks, et al 2003) Such mutations occur in the four exons at the 3' region of the gene which mainly encode the C2 domain (Eng, 2003). Deletion and translocation of PTEN have also been described in some cases (Marsh, et al 1999 & Ahmed, et al 1999). Once again, BRRS patients with PTEN mutations are more likely to develop breast cancers, and BRRS and CS are now regarded as phenotypic variations of the same syndrome. The causes of the remaining ~40% of BRRS cases without PTEN mutation are unknown (Dahia, 2000), but PTEN deletion has been detected in ~11% of these cases (Zhou, et al 2003b).

Lhermitte-Duclos Disease (LDD)

LDD is regarded as a neurological manifestation of CD, and is characterised by hamartomatous overgrowth of cerebellar ganglion cells, which replace granular and Purkinje cells (Vantomme, et al 2001). Patients suffer ataxia and seizures due to increased intracranial pressure, and PTEN haploinsufficiency is sufficient to mimic these symptoms in mice (Li, et al

(2003b). LDD is usually sporadic rather than familial, but it is unclear as to how PTEN mutations contribute to this disease, which has only recently been recognised as part of PHTS.

Zhou, et al (2003b), presented data suggesting a high incidence (83%) of adult-onset LDD cases had germline mutations in PTEN, resulting in little or no protein expression in 75% of the samples which also had elevated P-Akt. They also propose that LDD in children and adults have distinct underlying causes, as very few children diagnosed with the disease carry PTEN mutations. The majority of somatic PTEN mutations in LDD are truncating (Koch, et al 1999), but there have been reports of germline mutations in exon 5 more usually seen in CD patients, which reduce phosphatase activity (Sutphen, et al 1999 & Robinson & Cohen, 2000).

Proteus Syndrome (PS)

PS, like LDD, has only recently been included under the PHTS grouping and is a rare sporadic, hamartomatous disorder of vascular, skeletal, and soft tissues with mosaic distribution. Patients can present with asymmetry of the skull, body, limbs and partial gigantism of the hands or feet, hypertrophy of long bones, haemangiomas, lipomas, macrocephaly, and focal growths of the skin and vascular tissues (Bilkay, et al 2003). PS-like (PSL) cases show less pronounced symptoms, and are more stable with less sporadic outbreaks of abnormal growth (Zhou, et al 2000b). Up to 20% of PS cases and 50% of PSL cases harbour germline PTEN mutations (Eng, 2003). Mutations unique to PS, which are not seen in CD, BRRS or LDD are W211R and C211X, and in PSL the mutation M35T (Zhou, et al 2001c).

As previously mentioned, the various phenotypes of PHTS diseases can arise from the same PTEN mutations. Common mutations in CD, BRRS and PS are Q110X, R130X, R233X, R335X, (CpG dinucleotides in the phosphatase domain) and P246L (C2 domain), emphasising the importance of these regions for PTEN function. Mutations in the CpG islands could possibly increase promoter silencing by methylation, and in the C2 domain mutations can have profound effects on the stability of PTEN protein, as previously described.

1.4.2 Somatic Mutations

Over 300 somatic mutations of PTEN have now been described in various types of tumour, including endometrium, breast, prostate and melanomas. ~70% of mutations in PTEN are frameshift, missense or splice mutations which truncate the protein. Approximately half of the ~100 missense mutations, or ~23% of all mutations, are found in the exon 5 phosphatase domain and may affect activity. Additionally, the structural abnormality known as loss of heterozygosity (LOH), where mutations in one allele lead to the deletion of the other, has been detected in ~90% of tumours. LOH is difficult to accurately detect, therefore this figure may not be truly representative (Bonneau & Longy, 2000).

Glioblastoma (GB)

PTEN mutations have been described in several neurological tumours as a late event which correlates with tumour progression and aggressiveness (Davies, et al 1999b) rather than predisposition (Sanson, et al 1999).

Glioblastoma (GB) is the most common and most malignant form of astrocytic tumour in adults, with a survival rate of <1 year (Reis, et al 2000). Loss of PTEN is seen in ~20-30% of primary tumours (Kato, et al 2000, Zhou, et al 1999 & Backlund, et al 2003) and is often accompanied by amplification of EGFR and aberrations in the Rb1 pathway and p53 (Reis, et al, 2000). LOH and promoter methylation have also been observed in GB (Baeza, et al 2003).

The molecular mechanisms underlying this disease remain elusive, but it has been proposed that mutations in EGFR and p53 are very early events, and that disruption of the Rb1 pathway (e.g., pRb, CDK4), in conjunction with “second hit” loss of PTEN may be progression factors. This could provide a diagnostic tool for the early detection and treatment of this disease (Backlund, et al 2003, Krex, et al, 2003). Studies in PTEN knockout mice suggest that inhibitors of mTOR, a downstream target of Akt/PKB, correct abnormal growth and could be useful in the treatment of LDD and glioblastoma (Kwon, et al 2003).

Together with the phenotypes observed in LDD and various murine models, the crucial role of PTEN in the development of the CNS has been highlighted (Di Christofano, et al 1998). Expression of PTEN protein in human foetuses has been observed to be the highest in the central and peripheral nervous systems, and with less intensity in adult brains (Gimm, et al 2000b). PTEN appears to be important for orchestrating brain architecture, cell size and migration, and has a much more important role in the development and maintenance of this organ than previously thought (Li, et al 2003).

Prostate Cancer

As in GB, PTEN mutation is a late event in prostate cancer which correlates to increasing grade of the tumour. Abnormal PTEN has been observed in ~15% of primary tumours and ~60% of advanced prostate cancers, with a high LOH frequency of ~40% being related to metastatic potential (Fernandez & Eng, 2002 & Deocampo, et al 2003). Low expression of the CDKI p27 is also indicative of aggressiveness and poor outcome (Halvorsen, et al 2003). It is believed that promoter hypermethylation is more important in this cancer, and there have been many reports of PTEN silencing in prostate tumours (Whang, et al, 1998 & Rennie & Nelson, 1999). Additionally, as prostate cancers frequently display androgen independence (See Section 1.2.4.2), loss of PTEN function may facilitate the activation of AR signalling and progression to this state in prostate cancer (Snabboon, et al 2003).

A recently developed murine model of the precancerous prostatic intraepithelial neoplasia (PIN) may be useful in the further elucidation of the molecular mechanisms in prostate carcinoma (Abate-Shen, et al 2003). The mouse model carries a heterozygous mutation in the *NKX3.1* prostate-specific homeobox gene which is deleted in ~80% of early lesions. Heterozygous loss of PTEN in this model leads to androgen independence, invasiveness and metastasis also observed in advanced prostate adenocarcinoma.

Malignant Melanoma

Frequent PTEN mutations have also been described in ~30% of late-stage malignant melanomas (Guldberg, et al 1997) and up to ~60% in advanced metastatic disease (Birck, et al 2000), but PTEN loss again appears to be

absent in early tumours (Poetsch, et al 2001). LOH of the PTEN locus is also very common in advanced melanomas, affecting of 30-50% of tumours and is associated with poor prognosis (Robertson, et al 1998 & Healy, et al 1998).

Breast Cancer

Primary sporadic carcinomas of the breast also harbour PTEN mutation, but at a much lower level of <5% compared to the cancers listed above (Rhei, et al 1997), and is therefore not implicated in the early stages of this disease (Ueda, et al 1998). LOH is observed in ~40% of invasive carcinomas in conjunction with loss of oestrogen receptors and increasing tumour grade (Bose, et al 1998). PTEN does not appear to be involved in inheritable breast cancer unless Cowden disease is also present, where it causes a predisposition to early-onset breast cancer (Mincey, 2003 & Carroll, et al, 1999).

More recently, loss of protein expression has been studied by several groups. Decreased or lack of PTEN was observed in ~38-50% of metastatic tumours, and also correlated with loss of oestrogen receptors and invasiveness (Depowski, et al 2001 & Bose, et al 2002).

In primary advanced breast adenocarcinoma, ~33% of tumours had decreased or no PTEN protein with LOH (Perren, et al (1999). The evidence indicates that PTEN loss is a late event in breast carcinogenesis which correlates with increased progression (Bose, et al 2002), and mutation in other genes such as p53 and BRCA1/2 (Kurose, et al 2002 & Buchholz, et al 2002). Constitutive activation of erbB2 autocrine signalling and increased P-Akt/PKB has been observed in breast cancer cell lines negative for PTEN,

and may be one mechanism promoting progression in these cells (Nicholson, et al 2003).

Endometrial Carcinoma (EC)

Endometrial cancers are the most common gynaecological malignancy affecting ~188,000 females worldwide per year (WHO, 2003), and are now recognised as having the highest rate of somatic PTEN mutation (Tashiro, et al 1997). Endometrial carcinomas (EC) display ~30-50%, adenocarcinomas (EAC) 50-85% and ~20% of the precursor lesion endometrial hyperplasia (EH) exhibit PTEN mutations. The figures for EAC represent the highest PTEN mutation rate in primary tumours, with all the mutations being in tumours of the endometrioid (Type I) variety which accounts for 80-90% of all ECs (Mutter, et al 2000). The predisposition to EC via PTEN mutation has been demonstrated in knockout mice, which develop atypical hyperplasia, the endometrioid precursor to EC (Podyspanina, et al 1999).

Mutation of PTEN appears to be an early event in EC, as aberration in the gene can be detected in early hyperplasias, (Levine, et al 1998), and such mutations may provide a diagnostic marker for the disease (Mutter, et al 2000, Gao, et al 2003b & Ricci, et al 2003). PTEN loss and Akt phosphorylation are indicative of poor prognosis, and survival rates are lower in patients who are immunohistochemically PTEN-negative (Terakawa, et al 2003). LOH at the PTEN locus has also been reported to be present in ~20-50% of advanced endometrial carcinomas, and may indicate progression of the disease (Tsuda, et al 2002 & Toda, et al 2001). The current mutational spectra for PTEN in EC is shown in Table 2.

Some correlation has also recently been reported between the amplification of the oncogene cMYC and PTEN mutation in early and advanced EC. In PTEN⁺/cMYC⁻ tumours, PTEN mutations were most frequent in exons 1-5, and less frequent in exons 7-8 (66.7% and 33.3%, respectively). In contrast, in PTEN⁺/cMYC⁺ carcinomas the PTEN mutations were found mainly in exons 7-8 (85.7%). The group implicate subtle genetic variations and interaction with different PTEN mutations for the different subsets of EC (Konopka, et al 2003).

| Table 2 Mutation Spectrum of PTEN in Endometrial Cancers | | | | | |
|--|---------------------|--------------|---------------------|--------------|-----------|
| Exon/Intron | Nucleotide Exchange | Mutation | Protein/mRNA Change | 2nd Mutation | Histology |
| E1 | c.36insA | Frameshift | Stop at 43 | No LOH | EC |
| E1 | c.37delA | Frameshift | Stop at 23 | ? | EC |
| E1 | c.37A-->G | Missense | K37Q | ? | ECIII |
| E1 | c.45ins T | Frameshift | Stop at 43 | ? | EC |
| E1 | c.49C-->T | Nonsense | Q17X | LOH | EC |
| E1 | c.54delGGAT | Frameshift | Stop at 23 | Dble Mut | EC |
| E1 | c.59G-->A | Missense | G20E | LOH | EC |
| E1 | c.70G-->A | Missense | D24N | LOH | EC |
| E1 | c.70insC | Frameshift | Stop at 43 | LOH | EC |
| E1 | c.76A-->C | Missense | T76P | ? | ECIII |
| E2 | c.80delAT | Frameshift | Stop at 42 | ? | EC |
| E2 | c.89delC | Frameshift | Stop at 53 | ? | EC |
| E2 | c.94delATT | In-Frame Del | I32del | Dble Mut | EC |
| E2 | c.95delT | Frameshift | Stop at 53 | MIN | EC |
| E2 | c.96delT | Frameshift | Stop at 53 | ? | EC |
| E2 | c.97del3 | In-Frame Del | I33del | LOH | EC |
| E2 | c.97delA | Frameshift | Stop at 53 | No LOH | EC |
| E2 | c.97delATTG | Frameshift | Stop at 53 | ? | EC |
| E2 | c.98T-->G | Missense | I33S | No LOH | EC |
| E2 | c.118G-->T | Nonsense | E40X | ? | EC |
| I2 | IVS2-1G-->T | Splicing | | LOH | EC |
| E3 | c.165-6del17 | Splicing | | ? | ECI |
| E3 | c.170insT | Frameshift | Stop at 62 | ? | ECI |
| E3 | c.171ins38 | Frameshift | Stop at 98 | Dble Mut | EC |
| E3 | c.176C-->A | Nonsense | S59X | LOH | EC |
| E3 | c.184delA | Frameshift | Stop at 98 | Dble Mut | EC |

| | | | | | |
|----|-------------|--------------|-------------|----------|-----|
| E3 | c.190delCA | Frameshift | Stop at 72 | ? | ECI |
| E3 | c.202delTA | Frameshift | Stop at 72 | LOH | EC |
| E3 | c.202insTAT | Frameshift | Stop at 74 | ? | EC |
| E3 | c.202T-->C | Missense | Y68H | ? | AEH |
| E4 | c.218delAA | Frameshift | Stop at 76 | MIN | EC |
| E4 | c.223insA | Frameshift | Stop at 77 | MIN | EC |
| E4 | c.227delAT | Nonsense | Y76X | LOH | EC |
| E4 | c.242T-->G | Frameshift | F to C | ? | EC |
| I4 | IVS4+1G-->A | Splicing | | MIN | EC |
| E5 | c.276delT | Frameshift | Stop at 86 | Dble Mut | EC |
| E5 | c.269T-->C | Missense | F90S | Dble Mut | EC |
| E5 | c.275A-->G | Missense | D96G | LOH | EC |
| E5 | c.277C-->G | Missense | H93D | MIN | EC |
| E5 | c.277C-->T | Missense | H93Y | MIN | EC |
| E5 | c.284C-->T | Missense | P95L | MIN | AEH |
| E5 | c.284C-->T | Missense | P95L | MIN | EC |
| E5 | c.296insTA | Frameshift | Stop at 112 | ? | EC |
| E5 | c.309insT | Frameshift | Stop at 106 | ? | EC |
| E5 | c.309del11 | Frameshift | Stop 105 | ? | EH |
| E5 | c.319G-->T | Missense | D107Y | ? | EC |
| E5 | c.356T-->A | Missense | V119D | Dble Mut | EC |
| E5 | c.360del75 | In-Frame Del | | MIN | EC |
| E5 | c.367C-->T | Missense | H123Y | MIN | EC |
| E5 | c.370T-->A | Missense | C124S | ? | EC |
| E5 | c.382del14 | Frameshift | Stop at 159 | ? | AEH |
| E5 | c.386G-->A | Missense | G129E | ? | EC |
| E5 | c.387delA | Frameshift | Stop at 133 | ? | EC |
| E5 | c.388C-->G | Missense | R130G | ? | EC |
| E5 | c.388C-->G | Missense | R130G | Dble Mut | EC |
| E5 | c.388C-->G | Missense | R130G | MIN | EC |

| | | | | | |
|----|---------------|--------------|-------------|----------|-------|
| E5 | c.388C-->T | Nonsense | R130X | ? | EC |
| E5 | c.388C-->T | Nonsense | R130X | MIN | EC |
| E5 | c.388C-->T | Nonsense | R130X | Dble Mut | EH |
| E5 | c.389del7 | Frameshift | Stop at 131 | ? | AEH |
| E5 | c.389del9 | In-Frame Del | | ? | EC |
| E5 | c.389G-->A | Missense | R130Q | LOH | EC |
| E5 | c.389G-->T | Missense | R130Q | ? | AEH |
| E5 | c.389G-->T | Missense | R130L | ? | AEH |
| E5 | c.389G-->T | Missense | R130L | ? | EC |
| E5 | c.392del20 | Frameshift | Stop at 172 | ? | AEH |
| E5 | c.397G-->A | Missense | V133I | LOH | EC |
| E5 | c.405insA | Frameshift | Stop at 179 | Dble Mut | EC |
| E5 | c.405del19 | Frameshift | Stop at 140 | | ECIII |
| E5 | c.415delTT | Frameshift | Stop at 178 | ? | EH |
| E5 | c.416T-->G | Nonsense | L139X | ? | EH |
| E5 | c.445C-->A | Nonsense | Gln to Stop | ? | AEH |
| E5 | c.459T-->C | No Change | No Change | ? | EC |
| I5 | c.486insA | Frameshift | Stop at 166 | Dble Mut | EC |
| I5 | c.486ins8 | Frameshift | Stop at 169 | ? | AEH |
| E6 | IVS5+2delT | Splicing | | LOH | EC |
| E6 | c.491delA | Frameshift | Stop at 166 | ? | ECI |
| E6 | c.509G-->A | Missense | S170N | ? | EC |
| E6 | c.517C-->T | Missense | R173C | ? | AEH |
| E6 | c.517C-->T | Missense | R173C | ? | EC |
| E6 | c.517C-->T | Missense | R173C | LOH | EC |
| E6 | c.527T-->G | Nonsense | Y176X | Dble Mut | EC |
| E6 | c.530delATTAT | Frameshift | Stop at 178 | Dble Mut | EC |
| E6 | c.537del4 | Frameshift | Stop at 181 | ? | AEH |
| E6 | c.545insA | Frameshift | Stop at 189 | MIN | EC |
| E6 | c.559delG | Frameshift | Stop at 198 | ? | EC |

| | | | | | |
|----|---------------|------------|-------------|----------|-----|
| E6 | c.572T-->C | Missense | V191A | ? | EH |
| I6 | c.IVS6+1G-->T | Splicing | | LOH | EC |
| I6 | c.IVS6+1G-->T | Splicing | | MIN | EC |
| E7 | c.632insG | Frameshift | Stop at 242 | ? | ECI |
| E7 | c.640C-->T | Nonsense | Q214X | ? | EC |
| E7 | c.640C-->T | Nonsense | Q214X | LOH | EC |
| E7 | c.640C-->T | Nonsense | Q214X | MIN | EC |
| E7 | c.646G-->A | Missense | V216M | LOH | EC |
| E7 | c.654C-->A | Nonsense | C218X | Dble Mut | EC |
| E7 | c.667delA | Frameshift | Stop at 225 | ? | EC |
| E7 | c.697C-->T | Nonsense | R233X | Dble Mut | EC |
| E7 | c.697C-->T | Nonsense | R233X | ? | AEH |
| E7 | c.703G-->T | Missense | E235X | LOH | EC |
| E7 | c.710insAA | Frameshift | Stop at 242 | LOH | EC |
| E7 | c.721delT | Frameshift | Stop at 255 | LOH | EC |
| E7 | c.727del13 | Frameshift | Stop at 251 | ? | EC |
| E7 | c.733C-->T | Nonsense | Q245X | Dble Mut | EC |
| E7 | c.733del8insA | Frameshift | Stop at 253 | No LOH | EC |
| E7 | c.737insGT | Frameshift | Stop at 256 | ? | AEH |
| E7 | c.738delG | Frameshift | Stop at 255 | MIN | EC |
| E7 | c.750T-->A | Nonsense | C250X | Dble Mut | EC |
| E7 | c.750delTG | Frameshift | Stop at 251 | ? | ECI |
| E7 | c.762del14 | Frameshift | Stop at 298 | ? | EC |
| E7 | c.792del13 | Frameshift | Stop at 271 | No LOH | EC |
| E7 | c.795delA | Frameshift | Stop at 275 | ? | EC |
| E7 | c.795delA | Frameshift | Stop at 275 | Dble Mut | EC |
| E7 | c.795delA | Frameshift | Stop at 275 | LOH | EC |
| E7 | c.795delA | Frameshift | Stop at 275 | MIN | EC |
| E7 | c.799delAA | Frameshift | Stop at 296 | Dble Mut | EC |
| E7 | c.800insA | Frameshift | Stop at 297 | ? | ECI |

| | | | | | | |
|----|--|--------------|------------|-------------|----------|------|
| E7 | | c.800delA | Frameshift | Stop at 275 | ? | EC |
| E7 | | c.801insA | Frameshift | Stop at 297 | ? | EC |
| E7 | | c.802ins171 | Frameshift | | ? | EC |
| I7 | | INS7+1G-->T | Splicing | | LOH | EC |
| E8 | | c.820delT | Frameshift | Stop at 275 | ? | ECI |
| E8 | | c.850G-->A | Missense | Q to K | ? | EC |
| E8 | | c.863delA | Frameshift | Stop at 290 | Dble Mut | EC |
| E8 | | c.864del5 | Frameshift | Stop at 295 | ? | EC |
| E8 | | c.867delA | Frameshift | Stop at 290 | ? | ECII |
| E8 | | c.877G-->T | Nonsense | G293X | MIN | EC |
| E8 | | c.892C-->T | Nonsense | Q298X | LOH | EC |
| E8 | | c.885insA | Frameshift | Stop at 297 | ? | ECII |
| E8 | | c.895G-->T | Nonsense | Q299X | ? | ECII |
| E8 | | c.901del10 | Frameshift | Stop at 303 | No LOH | EC |
| E8 | | c.923delGT | Frameshift | Stop at 310 | ? | AEH |
| E8 | | c.937delA | Frameshift | Stop at 315 | ? | EC |
| E8 | | c.940insG | Frameshift | Stop at 324 | ? | ECI |
| E8 | | c.950del4 | Frameshift | Stop at 319 | Dble Mut | EC |
| E8 | | c.950del5 | Frameshift | Stop at 320 | MIN | EC |
| E8 | | c.952delCTTA | Frameshift | Stop 319 | ? | AEH |
| E8 | | c.952delCTTA | Frameshift | Stop 320 | Dble Mut | EC |
| E8 | | c.952delCTTA | Frameshift | Stop at 319 | Dble Mut | EC |
| E8 | | c.953delTTAC | Frameshift | Stop at 319 | ? | AEH |
| E8 | | c.955delACTT | Nonsense | T319X | LOH | EC |
| E8 | | c.955delACTT | Nonsense | T319X | MIN | EC |
| E8 | | c.956delCTTT | Frameshift | Stop at 342 | ? | EC |
| E8 | | c.956insA | Frameshift | Stop at 324 | Dble Mut | EC |
| E8 | | c.962insA | Frameshift | Stop at 324 | ? | EC |
| E8 | | c.962insA | Frameshift | Stop at 324 | Dble Mut | EC |
| E8 | | c.962insA | Frameshift | Stop at 324 | MIN | EC |

| | | | | | |
|----|-------------|--------------------|-------------|----------|------|
| E8 | c.962insA | Frameshift | Stop at 324 | No LOH | EC |
| E8 | c.963delA | Frameshift | Stop at 343 | ? | EC |
| E8 | c.963delA | Frameshift | Stop at 343 | Dble Mut | EC |
| E8 | c.963delA | Frameshift | Stop at 343 | MIN | EC |
| E8 | c.963delA | Frameshift | Stop at 343 | No LOH | EC |
| E8 | c.963delAA | Frameshift | Stop at 324 | ? | EC |
| E8 | c.968delA | Frameshift | Stop at 343 | ? | EH |
| E8 | c.968delA | Frameshift | Stop at 343 | ? | EC |
| E8 | c.968insA | Frameshift | Stop at 324 | ? | ECI |
| E8 | c.969insA | Frameshift | Stop at 324 | ? | EC |
| E8 | c.976del55 | In-Frame Del + Spl | | Dble Mut | EC |
| E8 | c.984del4 | Frameshift | Stop at 343 | ? | EC |
| E8 | c.987del4 | Frameshift | Stop at 343 | ? | EC |
| E8 | c.988delAA | Frameshift | Stop at 341 | ? | EC |
| E8 | c.992A-->G | Missense | D331G | No LOH | EC |
| E8 | c.999ins7 | Frameshift | Stop at 344 | ? | ECI |
| E8 | c.1003C-->T | Nonsense | R335X | ? | EC |
| E8 | c.1004del11 | Frameshift | Stop at 338 | ? | ECI |
| E8 | c.1008C-->A | Nonsense | Y336X | LOH | EC |
| E8 | c.1009delT | Frameshift | Stop at 343 | ? | EC |
| E8 | c.1012T-->A | Missense | S to T | | |
| E8 | c.1021T-->G | Missense | F to V | ? | ECII |
| E9 | c.1043C-->T | Missense | T348I | ? | AEH |
| E9 | c.1043insA | Frameshift | Stop at 360 | Dble Mut | EC |

Table 2. Mutation Spectrum of PTEN in Endometrial Cancers, Legend

Mutations are numbered according to the PTEN DNA code and starting from A in the start codon of the protein.

E: Exon, **I:** Intron, **del:** deletion, **ins:** insertion, **In-Frame Del:** In-Frame deletion.

AEH: atypical endometrial hyperplasia, **AH,** **EH:** endometrial hyperplasia, **EC (I/II/III):** endometrial carcinoma (Grades I/II/III) **LOH:** Loss of heterozygosity, **MIN:** microsatellite instability, **Dble mut:** Two point mutations in same tumour, **IVS:** intronic variant sequence, **?:** No information.

Table adapted from Bonneau & Longy (2000), Sun, et al (2001), & Minaguchi, et al (2001)

The location of mutations in PTEN also appears to have significance in EC. There have been several reports of PTEN mutations giving a better prognosis in endometrioid tumours (Risinger, et al 1998 & Maxwell, et al 2000), although the specific mutations were not analysed. Minaguchi, et al (2001) described better survival rates in patients with mutations outside of exons 5, 6 and 7, although the tumours were histopathologically indistinguishable from those with PTEN mutations inside these exons. They associated exon 8 mutation with a better prognosis as these mutations affect only the C2 domain and may still retain phosphatase activity (refer to Section 1.2.2). Exon 7 mutation was however correlated with poor outcome as most mutations here are frameshifts or nonsense, which lead to truncation of the CBR3 region of the phosphatase domain and the C2 domain. This mutation would be comparable to an exon 5 catalytic site mutation and most likely would disrupt PI3,4,5P3 binding.

In addition to mutation, PTEN promoter methylation has been detected in ~20% of EC lesions and correlated with increasing tumour grade and metastasis (Salvessen, et al 2001 & Risinger, et al 2003).

Microsatellite instability (MSI) due to replication errors occurs frequently in hereditary tumors and appears to be an early event in EC. MSI is caused by mutations in DNA mismatch repair genes hMLH1 and hMSH2, but errors are seen in hMLH1 more often in EC (Piero, et al 2002). Reports have detected loss of hMLH1 and PTEN expression in 55% of EC samples (Orbo, et al 2003), and also in endometriosis, which has the potential to become malignant (Martini, et al 2002). MSI has been associated with a favourable prognosis in endometrioid EC (Maxwell, et al 2001, Wong, et al 1999 &

Tibilietti, et al, 1999), and colorectal cancers (Gryfe, et al 2000). One possible reason for the more favourable prognosis is that lymphocytic invasion is higher in EC lesions with MSI, suggesting augmentation of host immune recognition of these tumours (Kihana, et al 1998).

PTEN mutations in EC could provide useful prognostic and therapeutic information, and aid in the decision to perform surgical removal of metastatic tumours or to offer treatments such as chemotherapy or radiotherapy. Additional studies on PTEN genetic alterations, regulation and cellular localisation in EC cells may help to clarify the tumor suppressor function of PTEN in this disease.

Other Malignancies

Aberrations of PTEN have now been reported in a wide range of other cancers, but the implications of PTEN mutation are less well understood in these diseases with regard to initiation and progression. Decreased PTEN expression has been detected in higher grade gastric cancer (Zheng, et al 2003b), colorectal cancer (Zhou, et al 2002), bladder (Aveyard, et al 1999) and pancreatic cancer (Perren, et al 2000).

PTEN mutation has also been analysed in haematological malignancies. Abberant PTEN has been detected in ~20% of acute myeloid leukaemias [AML] (Liu, et al 2000 & Aggerholm, et al 2000). The high incidence of loss of protein expression in the presence of high levels of PTEN mRNA in primary leukaemia and Non-Hodgkins' lymphoma indicate that gene silencing, probably by promoter methylation, is important in haematological cancers (Dahia, et al 1999). Recently, high levels of P-PTEN, P-Akt/PKB

and phosphorylated Forkhead factors have been detected in this disease indicating poor prognosis (Cheong, et al 2003a & b). Conversely, PTEN mutations are rarely seen in lymphoma (Melendez, et al 2003), although lower expression levels have been observed (Abbott, et al 2003).

Loss of PTEN may also play a role in autoimmune diseases, as it has been shown that in during clonal selection T-cells with PTEN deficiency become resistant to CD95-induced death and gain long-term activity (Strauss, et al 2003). Loss of PTEN also disrupts B-cell immunoglobulin class switching and differentiation in the spleen (Anzelon, et al 2003) and can increase autoantibody titres (Suzuki, et al (2003).

1.4.3 PTEN Promoter Methylation

Promoter methylation is an alternative method of silencing tumour suppressor genes (Herman, 1999) and has been observed in a variety of tumour types for many cancer-related genes including p16 (Yu, et al 2002), BRCA1 (Baldwin, et al 2000) and Rb (Simpson, et al 2000). Methylation occurs at 'CpG islands', dinucleotides which are usually present within or near the promoter. Hypermethylation by DNA methyltransferases prevents transcription of the downstream gene, and elevated expression of DNA methyltransferase I is frequently observed in cancers (Warnecke & Bestor, 2000).

Several papers have reported methylation of the PTEN promoter in a number of tumour cell types, including gastric carcinomas (Kang, et al 2002), endometrial carcinomas (Salvesen, et al 2001), thyroid cancers (Frisk, et al 2002) and prostate carcinomas (Whang, et al, 1998). Soria, et al (2002) also

report methylation of the PTEN promoter in non-small cell lung carcinoma (NSCLC) which often presents constitutive Akt/PKB. This group were able to demethylate PTEN in a NSCLC cell line with 5-aza-2'-deoxycytidine which restored mRNA transcription. They report that as demethylating agents are undergoing clinical trials, their finding is of particular therapeutic significance. It is noted by Zysman, et al (2002) that many of the methods used to determine the methylation status of the PTEN promoter cannot differentiate between PTEN and the pseudogene ψ PTEN which are discussed below.

1.5 The PTEN Pseudogene & Tissue-Specific PTEN Homologues

ψ PTEN, the Pseudogene

Shortly after the discovery of PTEN, a highly conserved processed pseudogene (Mighell, et al 2000), named ψ PTEN was located on human chromosome 9p21 (Dahia, et al 1998). ψ PTEN differed from PTEN by 19 nucleotides and 12 amino acids (see Appendix III), had no initiation methionine and was intronless. Despite these features, several groups have described varying levels of transcription of ψ PTEN mRNA in a range of tissues but were unable to detect any protein expression (Fujii, et al, 1999, Yokoyama, et al 2000).

PTEN Homologues TPTE, PTEN-2 and TPIP

Several PTEN homologues have now been reported in both human and mouse genomes. In 1999 a testis-specific conserved PTEN homologue called TPTE (transmembrane phosphatase and tensin homology) was cloned from human chromosome 21. Multiple copies also mapped to chromosomes

13, 15, 22 and Y, but this group did not characterise the protein (Chen, et al 1999). Work by Guipponi, et al (2000) has described the DNA structure of the copy on the short arm of chromosome 21 as having 24 exons spanning 87Kb. The same group also cloned murine TPTE, of which 3 major transcripts a, b, and c were described, each coding for proteins containing a protein tyrosine phosphatase motif and four potential transmembrane domains which localised to the golgi (Guipponi, et al 2001).

A highly conserved murine PTEN homologue, named PTEN 2, has recently been identified (Wu, et al (2001). This form appears to be expressed only in the testis and is localised to the golgi, possibly via an extended N-terminus containing four potential transmembrane domains. This association with the golgi may indicate a specialist role in membrane trafficking and regulation of terminal spermatocyte development. PTEN 2 also demonstrates enzymatic activity against PI3,4,5P3, and has a greater specificity for 3' and 5' phospholipids than PTEN although the reason for this is unclear.

Walker, et al, (2001) have also described another human PTEN homologue termed TPIP (TPTE and PTEN homologous inositol lipid phosphatase). This homologue has two splice variants, TPIP α and β , the former which has phosphatase activity and binds membranes, and the latter which is inactive and cytosolic. TPIP α contains up to three possible transmembrane domains that are absent in TPIP β , and localises to the endoplasmic reticulum (ER), where it may regulate phosphoinositide signalling. This group also demonstrated that TPTE was catalytically inactive against phospholipids, but protein phosphatase activity was not assessed. Additionally, none of the three homologues could regulate Akt/PKB.

1.6 Project Aims

Overview

Endometrial carcinoma is the most common gynaecological malignancy and has the highest rate of PTEN mutation over any other cancer. Over 100 mutations have been described, but none of the studies have been performed using archival endometrial material. A repository of ~50 patients was made available for use in this project, consisting of a histologically normal cervical sample and up to three tumour samples of varying histological grade. These samples permitted the analysis of the PTEN sequence in both normal and tumour cells. The role of TGF- β 1 in the regulation of PTEN levels is contradictory and may be cell-type specific, and has not yet been assessed in endometrial cells. Compounds which upregulate PTEN may have therapeutic value and therefore merit investigation. The subcellular localisation of PTEN has been reported to be both cytoplasmic and nuclear, and this may depend on both cell-type and stimulating compound. As PTEN levels respond to cyclic hormones in the endometrium, the cloning of PTEN into a plasmid encoding a green fluorescent protein tag (EGFP) would enable rapid study of PTEN protein behaviour in endometrial cell lines under hormone and cytokine stimulation. Specific project aims are detailed overleaf.

Specific Aims

The major aims of this project were threefold:

1. To analyse PTEN mRNA and protein expression in endometrial cell lines stimulated with TGF- β 1.

The transcriptional and post-translational effects of this cytokine were evaluated in two cell lines of endometrial origin, HEC-1B and Ishikawa, to compare the response of cells with wild-type PTEN (HEC-1B) or mutant PTEN (Ishikawa). Expression of PTEN, phospho-PTEN and several other genes were studied using RT-PCR and Western blot to determine the nature of any changes in expression produced by exposure to TGF- β 1. Expression levels of PTEN mRNA and protein were correlated to time and dose of cytokine stimulation. As serum in the growth medium can contain varying levels of TGF- β 1, stimulation of endometrial cell lines with cytokine was also performed in low-serum conditions and the results compared to normal serum conditions. The effect of TGF- β 1 on cell morphology and viability was also investigated.

2. To determine the sub-cellular localisation of PTEN protein in endometrial cell lines.

Localisation of PTEN protein has not previously been described in cell lines of endometrial origin. To assess localisation of PTEN in endometrial cell lines, the cloning of full-length human PTEN tagged with green fluorescent protein (GFP) was undertaken. Cell lines were transfected with PTEN-GFP and the localisation of the protein to cellular compartments observed. The effect of treatment with compounds reported to change PTEN expression

(TGF- β 1 and oestrogen) were also evaluated to determine whether the localisation of PTEN could be altered by such compounds.

3. To detect novel mutations of PTEN exons 5 and 8 in DNA derived from archival endometrial material.

Mutation of PTEN is especially prevalent in endometrial cancer, and by describing such mutations the specific role of PTEN in this disease can be analysed. Using PCR fragments amplified from tumour DNA for PTEN exons 5 and 8, the presence of sequence variations in the samples were detected by Single-Strand Conformation Polymorphism (SSCP) analysis. Samples with sequences differing from a PTEN control sample were detected as bands with aberrant mobility. Samples displaying such bandshifts may contain polymorphisms or mutations, which can be determined by automated sequencing of the PCR products.

CHAPTER 2

MATERIALS & METHODS

2.1 MATERIALS & SUPPLIERS

2.1.1 Reagents & Kits

McCoy's 5A Modified Medium, (M8403), Sigma

Minimal Essential Medium Eagle's, (M2289), Sigma

Human recombinant TGF β -1, (240-B), R&D Systems, UK

Protease Inhibitor Cocktail for Mammalian Cells, (P8340), Sigma

Bradford Reagent, (B6916), Sigma

Colour SDS Molecular Weight Markers, High Range, (C3312), Sigma

Biotinylated SDS Molecular Weight Markers, (MW-SDS-100B), Sigma

ECL Blocking Powder, Amersham

Western Blotting Detection System, (RPN 2209), Amersham

CellTiter One Solution Cell Proliferation Assay Solution, (G3580), Promega

Normal Goat Serum, (G9023), Sigma

GenElute Plasmid Miniprep Kit, (PLN-10), Sigma

GeneRuler 1Kb molecular weight marker 0.25mg/ml, (SM0313), MBI
Fermentas

Poly-D-Lysine, (P7405), Sigma

DAPI, (D8417), Sigma

Fluoromount G, (0100-01), Southern Biotechnology

Lipofectamine, (18324), Lipofectamine 2000, (11668), Invitrogen

β -17 Estradiol (E8875) Sigma

CMV-PEGFP-N1 Courtesy of Dr E Blair, Dept Molecular Biology, University of Leeds, UK

CMV-pECFP-N1 Courtesy of Dr C Fenech, Sheffield Hallam University, UK

2x MDE gel solution, (50620), Bio Whittaker Molecular Applications

PlusOne DNA Silver Staining Kit, (17-6000-30), Amersham Pharmacia Biotech

100bp Molecular Weight Marker, (N3231S), NEB

Sequencing performed to order by:

Lark Technologies Inc

Radwinter Road

Saffron Waldon

Essex CB11 3HY

2.1.2 Antibodies

Mouse monoclonal IgG1 anti-PTEN, Clone A2B1, 0.5 mg/ml, (68731A), BD Transduction Laboratories

Mouse monoclonal IgG anti-PTEN, Clone 6H2.1, 0.5 mg/ml Alexis Biochemicals

Mouse monoclonal anti- β -Actin, Clone AC15, (A5441), Sigma

Mouse monoclonal IgG1 anti-PAI-1, 0.25 mg/ml, (612024), BD Transduction Laboratories

Mouse monoclonal IgG1 anti-p27KIP1, Clone G-173-524, 0.5 mg/ml, (554069), BD Transduction Laboratories

Mouse monoclonal IgG2a anti-Phospho-AKT Ser 473, Clone 4E2, (#9276), NEB

Rabbit Polyclonal anti-Phospho-PTEN Ser 380, (#9551) NEB

Vector Elite anti-mouse IgG detection kit & anti-Universal detection kit, Vector Laboratories

AlexaFluor 594 goat anti-mouse IgG (H+L) Highly cross-adsorbed, 2 mg/ml, (A-11005), Molecular Probes

Anti-Hsp47, (Colligin), mouse monoclonal 0.85mg/ml, (SPA-470), Stressgen

Anti-Mannosidase II, Courtesy of Prof D Parkinson, Sheffield Hallam University

2.1.3 Oligonucleotides

The oligonucleotides used for PCR were synthesized by Sigma-Genosys with HPLC purification. Lyophilised oligonucleotides were re-suspended in 0.5-1ml of sterile water, aliquoted and stored at -20°C . The location of each PTEN primer is illustrated in Appendix II.2.

PTEN 1[#] Forward Exonic

5' CGGCATATGACAGCCATCATCAAAGAGATC3'

PTEN 2a[#] Reverse Exonic

5' CCC GTC GAC TCA GAC TTT TGT AAT TTG TG 3'

PTEN 3[#] Forward Exonic

5' CGG CAT ATG TGT GAT CAA GAA ATC GAT AGC 3'

PTEN 4[#] Reverse Exonic

5' CCCGTCGACTCACCCATAGAATCTAGGG3'

PTEN 5A* Forward intronic

5' TATTCTGAGGTTATCTTTTA 3'

PTEN 5B* Reverse exonic

5' CTTTCCAGCTTTACAGTGAA 3'

PTEN 5C* Forward exonic

5' GCTAAGTGAAGATGACAATCA 3'

PTEN 5D* Reverse intronic

5' AGAAAAACATCAAAAATAA 3'

PTEN 8A* Forward intronic

5' ACACATCACATACATAAAGTC 3'

PTEN 8B* Reverse Exonic

5' GTGCAGATAATGACAAGGAATA 3'

PTEN 8C* Forward Intronic

5' TTAAATATGTCATTTTCATTTCTTTTTC3'

PTEN 8D* Reverse Exonic

5' CTTTGTCTTTATTTGCTTTGT 3'

PTEN 10# Forward Exonic

5' CCC **GTC GAC** GGA CTT TTG TAA TTT GTG TAT 3'

PTEN 11^{dl} Reverse Exonic

GGC GAA TTC AGC ATG ACA GCC ATC ATC AAA GAG 3'

PTEN 10b# Forward Exonic

5' CAC CAC CTG TCG **ACT** GGA CTT TTG TAA TTT GTG TAT 3'

PTEN11a^μ Reverse Exonic

5' CGG ACT GAA **TTC** AGC ATG ACA GCC ATC ATC AAA GAG 3'

(*Sa*/I sites are highlighted in red and the *Eco*RI sites in blue).

GAPDH 472F7 Forward Exonic

5' TGA TGA CAT CAA GAA GGT GGT GAA G 3'

GAPDH 472R/ Reverse Exonic

5' TCC TTG GAG GCC ATG TGA GGC CAT G 3'

PAI-1F* Forward Exonic

5' GCT GAA TTC CTG GAG CTC AG 3'

PAMR* Reverse Exonic

5' CTG CGC CAC CTG CTG AAA CA 3'

TPIPF[†] Forward Exonic

5' TAT TCT GAT AAG AAT TTT TCA TCT GC 3'

TPIPR[†] Reverse Exonic

5' CCT CGG CAG TTA AAA ATA TTT CG 3'

Bcl-2A[°] Forward Exonic

5' GTT CGG TGG GGT CAT GTG TGT GGA GAG CG 3'

Bcl-2B[°] Reverse Exonic

5' TAG CTG ATT CGA CGT TTT GCC TGA 3'

Denotes primer sequence taken from *Risinger *et al* (1997), [†]Walker, *et al*, (2001). All other primers designed from Entrez sequences [#]AF067844, [‡]M16006, [§]BC040499, [‡]X05744, [°]M14745, ^ƒNM002046.

2.1.4 Enzymes

Red Hot Taq Polymerase, 5U/μl, (AB 0406/B), ABGene

Superscript II RNase H- Reverse Transcriptase, 200U/μl, (18064-014),
Invitrogen

Placental RNase Inhibitor, 50,000U/ml, (R2520), Sigma

Platinum *Pfx* DNA Polymerase, Invitrogen

Sal I, 20,000 U/ml, (R0138S), NEB

Eco RI, 20,000 U/ml, (R0101S), NEB

Mse I, 10,000 U/ml, (R0525S), NEB

Hha I, 20,000 U/ml, (R01395), NEB

T4 DNA Ligase, 400,000 U/ml, (M0202S), NEB

RNase A, (R6513), Sigma

2.1.5 Bacterial Strains and Cell Lines

E. coli JM109 courtesy of Dr A. Fairclough, Sheffield Hallam University

E. coli DH5α Subcloning Efficiency Chemically Competent Cells, (18265-
017), Invitrogen

HEC-1B cell line courtesy of Dr Sue Laird, Sheffield Hallam University, UK

Ishikawa cell line purchased from ECACC (99040201)

2.2 ANALYSIS OF EXPRESSION OF PTEN AND SELECTED GENES IN HEC-1B & ISHIKAWA CELL LINES STIMULATED WITH TGF- β 1

To assess the effect of TGF- β 1 on mRNA expression in HEC-1B and Ishikawa cells, RT-PCR was used to amplify mRNA species for PTEN, GAPDH and PAI-1.

2.2.1 Cell culture

Human endometrial cell lines HEC-1B and Ishikawa were routinely maintained in McCoy's 5A and Minimal Essential Eagles medium respectively (see Appendix I.1), at 37°C in 5% CO₂ atmosphere. Cells were passaged at a ratio of 1:10 every three to four days when 80% confluence was reached. The growth medium was discarded, 5 ml of Trypsin-EDTA solution added and cells were incubated at 37°C until they detached from the flask surface. Trypsinised cells (0.5ml) were then diluted in 9.5 ml of fresh complete medium in a new flask and returned to the incubator.

2.2.2 TGF- β 1 stimulation

To analyse the effect of TGF- β 1 on gene expression in HEC-1B and Ishikawa cell lines, cell populations were expanded in 250 ml flasks, prior to passaging 5ml of cells into 75 ml flasks. The cells were grown to 100% confluence in complete medium then washed once with fresh complete medium. For serum-starved samples the cells were washed with reduced-serum medium (Appendix I.1) and incubated in reduced-serum media for 24 hours.

Solutions of TGF- β 1 at concentrations of 2ng/ml and 5ng/ml of TGF- β 1 were freshly prepared from a sterile 2 μ g/ml stock of the human recombinant cytokine by diluting with complete or reduced-serum medium. Concentrations were chosen based on previous work by Lee, et al (1999). Medium was discarded from the cells and replaced with medium containing TGF- β 1 at the appropriate concentration, then cells were returned to the incubator for four, eight or twenty-four hours prior to RNA extraction. Duplicate flasks were prepared for each concentration and time, and controls included cells without TGF- β 1 at each time point and a pre-starvation '10% serum' sample for the serum-starved experiments.

2.2.3 Analysis of RNA Expression

2.2.3.1 RNA Extraction

Medium was discarded from each flask and the cells washed with sterile 1x PBS. The PBS was discarded and 2.5ml cold TRI Reagent (Sigma) added per flask in accordance with the manufacturers' recommendations. The cells were resuspended by repeated pipetting and immediately transferred to sterile Eppendorf tubes, using two tubes per sample. The cells were incubated on ice for 5 minutes to lyse before the addition of 150 μ l of ice-cold chloroform and a further incubation on ice for 10 minutes. The samples were centrifuged at 12,000 rpm for 15 minutes at 4°C in a Heraeus microcentrifuge and the upper layer transferred to fresh sterile tubes. An equal volume of ice-cold isopropanol was added to each sample and the tubes incubated for 20 minutes at -70°C.

The RNA was pelleted by centrifugation at 12,000 rpm for 15 minutes at 4°C and the supernatant discarded. The pellets were washed with 375µl of ice-cold 80% ethanol, centrifuged at 10,000 rpm for 5 minutes at 4°C and the supernatant carefully aspirated. The RNA samples were air dried at room temperature for 10 minutes and dissolved in 20µl of ice-cold sterile water and immediately stored at -70°C.

2.2.3.2 RNA Quantitation and RT-PCR

For each RNA sample 2.5µl aliquots of RNA were added to 497.5µl of ice-cold sterile water to give a dilution of 1:200 and stored on ice. The absorbance at 260nm and 280nm was determined for each sample on a Cecil Aquarius spectrophotometer, using water to zero the machine at each wavelength. Readings were obtained from the spectrophotometer for each sample for the concentration at 260nm, and the purity ratio of 260 / 280 as calculated by the machine. Measurements were performed in duplicate. Each sample was adjusted to a concentration of 1µg/µl with ice-cold sterile water.

2.2.3.3 cDNA Synthesis

For cDNA synthesis, Superscript II RNAase H- RT enzyme (Invitrogen) was used according to the manufacturers' instructions. This enzyme lacks the ability for RNA hydrolysis, thus increasing product yield (www.invitrogen.com). Briefly, a master mixture containing 3µl 5mM dNTPs, 3µl 0.1M DTT, 6µl 5x RT buffer, 0.5µl Oligo (d)₁₆T and 14.5µl sterile water per reaction was prepared on ice and mixed by brief vortexing. Aliquots of 27µl were added to each reaction tube. For each RNA sample two reactions

were prepared, consisting of a positive 'cDNA' containing 1µl of RNA (1µg), and a '-RT' also containing 1µl of RNA. An additional reaction tube without RNA (-RNA) was included in which sterile water (1µl) replaced RNA. The samples were mixed and heated at 65°C for 5 minutes on a thermal cycler, then immediately transferred to ice for 3 minutes. To each tube 1µl of placental RNase Inhibitor and Reverse Transcriptase were added; 1µl of sterile water was pipetted into the tubes labeled '-RT' in the place of RT. The samples were then incubated at 42°C for 1 hour on a thermal cycler and the cDNAs and controls stored at -20°C.

2.2.3.4 PCR

PCR was performed using cDNA diluted 1:4 with sterile water and a master mixture containing 2.5µl 10x Taq Buffer, 2.5µl 2mM dNTPs, 1µl 50mM MgCl₂, 100pM forward primer, 100pM reverse primer, 15µl sterile water and 1µl BioTaq Red DNA Polymerase per reaction. The master mixture was mixed by flicking gently and 24µl was aliquoted into each reaction tube, to which 1µl of diluted cDNA was added. The PCR reactions were mixed gently, briefly microfuged and placed in the thermal cycler on the appropriate programme (Appendix V). Samples were stored at 4°C when the PCR cycle had ended. PCR products were analysed by electrophoresis of 10µl aliquots using a 2% agarose gels as described below. Samples were loaded as a time-course for each concentration of TGF-β1 and control.

2.2.3.5 Analytical Scale Agarose Gel Electrophoresis of DNA

Analytical agarose gels were prepared by adding 300mg or 600mg of molecular grade agarose to 30ml of 1x TAE buffer (Appendix I.8) to give 1% or 2% gels respectively (Maniatis, Sambrook & Fritsch, 1989). The gel slurry was heated for 1 minute in a microwave oven on full power until the agarose had fully dissolved, and then allowed to cool briefly before the addition of 1.5 μ l of 10mg/ml ethidium bromide solution. The molten agarose was poured into a gel former with a comb inserted and allowed to set for 30 minutes at room temperature.

The comb was removed from the prepared gel which was then placed in a mini-submarine electrophoresis tank and submerged in 1xTAE buffer to the manufacturer's indicated mark. 10 μ l of PCR product was added to 2 μ l of loading dye (Appendix I.8) and the entire sample loaded through the buffer into a pre-formed well of the gel. 1 μ l of 100bp molecular weight marker was also included in one well. Electrophoresis was carried out at a constant 110V for approximately 45 minutes and the gel visualized on a UV transilluminator and photographed using a KODAK DC120 digital camera.

2.2.3.6 Densitometric Analysis of RT-PCR products

Gels were analysed using Kodak 1D Image Analysis Software (Version 2.0.1) using a 100bp molecular weight marker as a standard for mass calculations, and the band intensities expressed in nanograms.

2.2.4 Analysis of Protein Levels in HEC-1B and Ishikawa Cell Lines stimulated with TGF- β 1

2.2.4.1 Protein Extraction and Quantification

For preparation of whole-cell extracts, TGF- β 1 stimulation was carried out in 75 ml cell culture flasks as described in section 2.2. Following stimulation, the flasks were removed from the incubator, the medium discarded and the cells washed twice with ice-cold sterile PBS. The PBS was discarded and 750 μ l of ice-cold phosphoprotein lysis buffer (Appendix 1.2) added to each flask, with brief homogenization by pipetting. The cell lysates were transferred to sterile 1.5 ml Eppendorf tubes and incubated on ice for 30 minutes to allow complete lysis of the cells. The lysates were cleared of insoluble material by microfuging for 10 mins at 13,000 rpm at 4°C and the supernatant transferred to clean tubes on ice.

2.2.4.2 Determination of Protein Concentration by the Bradford Assay

Protein concentration in each sample was determined using the Bradford Assay. This simple, rapid method quantitates the amount of protein in a sample by the proportional binding of coomassie blue dye to the protein, which can be measured at an absorbance of 595nm (Friedenauer & Berlet, 1989). For each sample, 5 μ l was diluted in 995 μ l of sterile water to give a 1:200 dilution; 500 μ l of these dilutions were then transferred to clean 1.5ml Eppendorf tubes and 500 μ l of Bradford Reagent (Sigma) added to each tube. The reactions were vortexed and incubated at room temperature for five

minutes, prior to recording the absorbance of each sample at 595 nm using disposable plastic cuvettes. All samples were prepared in duplicate.

Average absorbance values were used to calculate the concentration of protein in the undiluted samples by reading from the BSA standard curve (Appendix IV) and adjusting for the dilution factor. The volume of each sample required to give 10 µg of protein was calculated and aliquots prepared for storage at -20°C.

2.2.4.3 Separation of Proteins by SDS-PAGE

SDS-polyacrylamide gels were prepared based on the Laemmli two-phase system of protein separation (Laemmli, 1970) using the Bio-Rad minigel (8x10cm) format system. Proteins separated by this method are denatured to single strands by the presence of SDS, which exposes the charged amino acid residues. Exposure of these residues permits electrotransfer of the proteins to a membrane, and subsequent detection by western blotting using antibodies.

The glass plates were cleaned with ethanol prior to assembly and an 8% resolving gel mix (Appendix 1.3) pipetted between the plates. The gel was overlayed with ethanol and allowed to polymerize at room temperature for one hour. The ethanol overlay was then discarded and the surface of the gel washed six times with distilled water and freshly prepared 4% stacking gel mix (see Appendix 1.3) pipetted on top of the resolving gel. A 10-well Teflon comb was carefully inserted into each stacking gel, avoiding air bubbles, and pressed down into the solution to give approximately 5 mm of stacking gel between the bottom of the wells and the resolving gel interface.

The stacking gel was left to polymerize for 30 minutes, then the combs carefully removed and the wells washed thoroughly with 1x Running buffer (Appendix I.3). The prepared gels were clamped into the electrophoresis module and both anode and cathode chambers filled with 1x Running buffer according to the manufacturer's recommendations.

Protein samples were prepared by mixing 10 µg of each sample with 1µl of loading buffer (Appendix I.9) and boiling at 100°C for 1 minute on a thermal cycler, with the lid heater set at 110°C. A coloured molecular weight marker was also boiled at 100°C for 1 minute, according to the manufacturer's instructions.

The entire volume of each sample was loaded into the wells of the SDS-PAGE gel using gel loading pipette tips, ensuring that the samples sank to the bottom of each well. To one well of each gel was added 4 µl of boiled colour marker and 0.5 µl of a biotinylated molecular weight marker (Appendix II). Gels were connected to a power supply and electrophoresed at 120 V for 90 minutes.

2.2.4.4 Electrotransfer of Proteins to PVDF Membrane

Following separation of proteins by SDS-PAGE, the gels were carefully removed from the tank and released from the glass plates into a volume of ice-cold Towbin Buffer (Appendix II.3) sufficient to cover the gel, in separate containers. An 8x10 cm piece of PVDF membrane was cut for each gel, pre-wet for a few seconds in methanol and submerged in Towbin buffer. The gels and PVDF were allowed to equilibrate on the bench for 15 minutes; the fiber

pads and blotting paper sheets were also wetted with buffer for the same period of time.

The transfer was set up as shown below in Figure 7.

Blotting Sandwich



Figure 7 Western blotting transfer assembly

The assembled sandwiches were inserted into the blotting module and placed into the tank along with a small magnetic stirrer bar and an ice pack. The tank was filled with Towbin buffer to the manufacturer's recommended level, the lid and electrodes attached and electrotransfer performed at 150V for 1 hour with stirring (transfer protocol modified from www.bdbiosciences.com/pharmlngen/protocols) After the transfer was completed, the PVDF membranes were removed from the sandwiches and air dried on paper towels. To assess the efficiency of the transfer, a gel was carefully retrieved and equilibrated in destain solution for 15 minutes prior to staining with coomassie blue overnight (Appendix 13). The stained gel was then destained to show the presence of any residual protein in the gel.

2.2.4.5 Detection of Proteins by Western Blot and Enhanced Chemiluminescence

PVDF membranes which had been electroblotted were pre-wet with methanol for a few seconds, then washed 3 times with T/P-BST, (Appendix II), for 5 minutes per wash. A sufficient volume of buffer was used to completely cover the membrane, and gentle shaking was applied during all stages of the Western process. The colour marker, placed to the left at all times, was used to orientate the membrane so that the proteins faced upwards. The Western protocol was modified from existing protocols (www.bdbiosciences.com/pharmingen/protocols and Maniatis, Sambrook & Fritsch, 1989). Membranes were incubated in the blocking buffer (Appendix I.3) for 1 hour at room temperature, then washed 3 times as previously described. The primary monoclonal antibodies were diluted in 1% BSA/PBST at 1:300 (α -PTEN), 1:20,000 (α - β -Actin), and 1:2000 (α -PAI-1). The blots were incubated with primary antibodies at room temperature for 90 minutes, washed three times, and incubated with anti-mouse secondary antibody diluted to 1:800 in 1% BSA/PBST at room temperature for 40 minutes. Anti-phospho-PTEN antibodies were used according to the manufacturer's instructions. The membranes for all primary antibodies were washed three times prior to incubation with Vector ABC reagent (avidin-biotin-horseradish peroxidase complex), prepared according to the manufacturer's instructions, for 30 minutes and again washed three times.

The ECL reagent was prepared according to the instructions supplied and applied to the membrane for 1 minute. Excess reagent was removed by touching the corners of the membranes with absorbent tissue, and the

membranes quickly sandwiched between two sheets of cling film with the colour marker to the left. Membranes were taken to the darkroom and exposed to film for 1 minute before developing according to the instructions supplied with the film and manual processing chemicals.

2.2.4.5.1 Densitometric Analysis of Protein levels

Photographic films which had been produced by the method described in 2.2.3 were scanned by Mr R Stewart, Academic Unit of Pathology, University of Sheffield and scanning densitometric analysis performed. The software used in the analysis was QuantityOne version 2.4 from the PDI Discovery package (Pharmacia).

2.2.5 HEC-1B Cell Proliferation Assay

To assess the effect of TGF- β 1 on HEC-1B cell proliferation, a Cell Titer 96 Aqueous One Solution Cell Proliferation Assay was used, according to the protocol. This MTT-based assay measures the cellular reduction of the assay reagent to a blue formazan product, which can only be performed by metabolically active cells (Mosmann, 1989). Briefly, cells were seeded on three 96 well plates corresponding to 4, 8 and 24 hours stimulation and grown to confluence in complete medium (Appendix I.1). Cells were serum-deprived in reduced-serum medium (Appendix I.1) for 24 hours and then stimulated with 0, 1, 2, 4, 5, 7 and 10 ng/ml TGF- β 1 in reduced serum medium. Controls either consisted of unstimulated cells prior to serum-starvation or they did not contain cells (growth medium and assay solution alone). Four replica wells were performed for each condition. Results were

obtained by reading the absorbance of the wells at 490nm in an automated plate reader, 1 hour after the addition of the assay solution.

2.2.6 Assessment of the effect of cell density on PTEN expression in HEC-1B cells.

To determine the effect of increasing cell density on PTEN expression levels in the HEC-1B cell line, cells were seeded at 4.5×10^5 and 9×10^5 cells per small (T25) culture flask. Cells were grown in complete medium for 1, 2, 3 and 4 days, plus 4 days with a growth medium change on day 3 (modified from Boyd & Kaufman, (1990). RNA extraction, cDNA synthesis and PCR were performed as described in Section 2.2.3. PCR products were analysed by running 10 μ l of each sample on a 2% agarose gels.

2.2.7 Morphological changes in HEC-1B and Ishikawa cell lines in response to TGF- β 1

The effect of TGF- β 1 on the morphology of HEC-1B and Ishikawa cells was investigated using a modified protocol previously described by Boyd & Kaufman, (1990). The cells were plated in triplicate onto two 24 well culture plates at an initial density of 1×10^4 cells/well in 1ml of complete medium. Cells were allowed to attach for 24 hours, then they were rinsed twice with reduced-serum medium (Appendix I.1) and maintained in this medium for 48 hours to induce synchronization. The medium was replaced with fresh reduced-serum medium containing 10ng/ml TGF- β 1 or an equivalent volume of vehicle, (4mM sterile HCL with 1% BSA). The medium containing TGF- β 1 or vehicle was replaced daily.

The morphology was observed after 48 and 72 hours by taking phase contrast light micrographs at x 200 magnification with a Leica DM/L inverted microscope using a Polaroid MicroSLR unit and 661 film. After photographing the cells, a cell count was performed by washing each well twice with PBS, trypsinising for 15 minutes and resuspending the cells in 1ml of complete medium. Cells were counted on a haemocytometer and the triplicate results shown graphically.

2.3 CLONING OF FULL LENGTH HUMAN PTEN INTO CMV-pEGFP-N1 & ANALYSIS OF SUBCELLULAR LOCALISATION.

2.3.1 Cloning Strategy

PCR primers were designed to amplify full length PTEN from cDNA. These primers, the sequences of which are shown in Section 3.1, were designed to add restriction endonuclease sites to the PCR product for directional cloning into the CMV-pEGFP-N1 vector (Figure 8).

The forward primer, (PTEN 10), contained a 5' *EcoRI* site (blue), and the reverse primer, (PTEN 11), contained a 5' *SalI* site (red). This should promote the ligation of double digested insert into pEGFP vector in one orientation only, and thus ensure that the correct strand of the cloned PTEN cDNA is transcribed. The overall cloning strategy is illustrated in Figure 9.



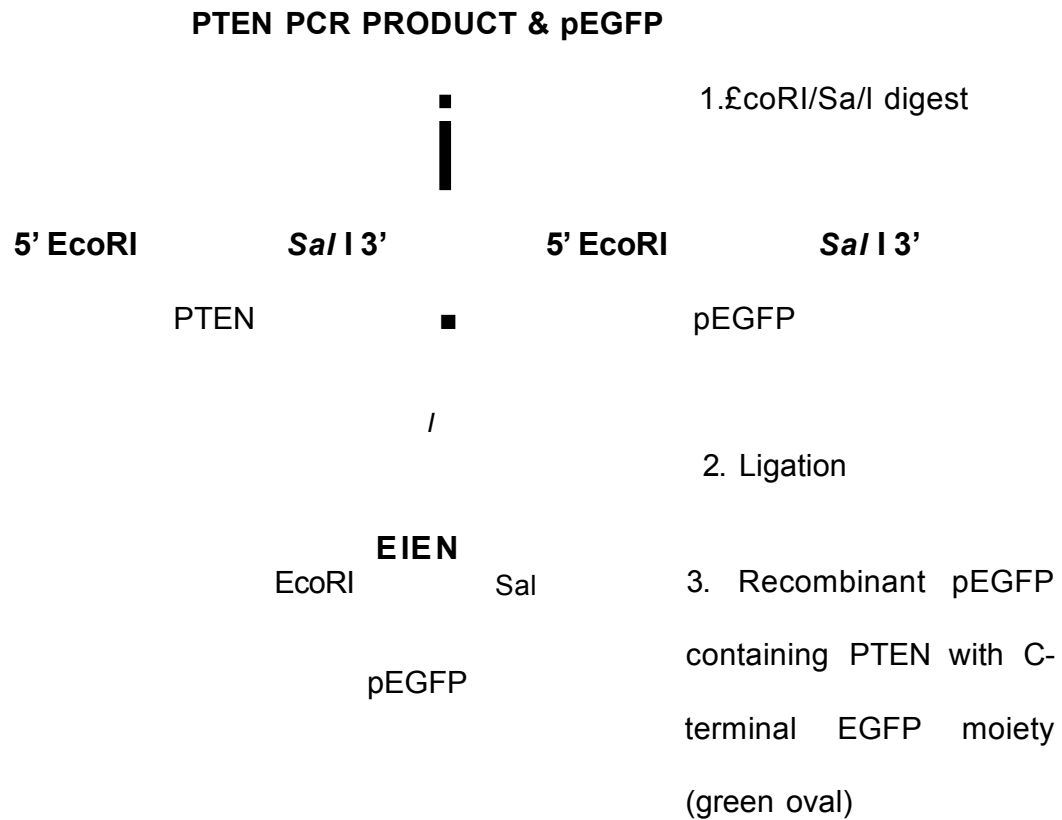


Figure 9

Cloning strategy showing 1. Digestion of PTEN & pEGFP, 2. Ligation of cut fragments to produce 3. Recombinant directional clone

2.3.2 Extraction of RNA from whole human blood

Fresh human venous blood was collected from four staff members into vacutubes containing EDTA and the tubes marked anonymously. Mononuclear cells were harvested using Histopaque (Sigma), according to the manufacturers' guidelines. Briefly, 5 ml of Histopaque was added to four sterile 15 ml Falcon tubes and carefully overlaid with 5 ml of each blood sample, ensuring that the blood did not mix with the Histopaque. The blood samples were centrifuged at 19,000 rpm for 30 minutes at room temperature using a Sorvall RT7 Plus centrifuge, and the mononuclear cells carefully collected from the interface using a sterile Pasteur pipette into clean 15 ml Falcon tubes.

The mononuclear cells were washed by adding 10 ml of sterile 1x PBS (Appendix I.3) followed by centrifugation at 2000 rpm for 5 minutes at room temperature using a Sorvall rotor. This step was repeated and the pelleted cells resuspended in 1 ml of cold TRI reagent for RNA isolation. Quantitation of the resulting RNA was performed as described in Section 2.2.3.

2.3.3 RT-PCR of Full Length Human PTEN for Cloning

2.3.3.1 Analytical Scale Digestion of cDNA With Restriction Endonucleases *Mse* I and *Hha* I

cDNA synthesis was performed using 5µg of total RNA isolated from each of the four members of staff, as described in 2.2.3.3 To verify that the cDNA produced was free of genomic DNA contamination, each cDNA sample was amplified by PCR with exonic primers PTEN 1 and 4, using the PTEN I PCR programme and conditions previously described in Chapter 2. These primers

amplified the N-terminal region of PTEN, yielding a fragment of 450bp in length which could be cleaved by *Mse* I and *Hha* I restriction endonucleases to distinguish between PTEN from mRNA or contaminating genomic Ψ PTEN pseudogene (Liu & Kagan, 1999).

PTEN derived from cDNA contains a unique *Mse* I site, and the pseudogene contains a unique *Hha* I site within the PCR fragment (Appendix III). Each of the PCR products were therefore subjected to a restriction digest with each enzyme in separate reactions. 5 μ l of PCR product was mixed in a sterile 0.2 ml Eppendorf tube with 1 μ l of the appropriate 10x restriction buffer, (supplied with each enzyme), and 3 μ l of sterile water. 1 μ l of the appropriate enzyme was added (10U of *Mse*I or 20U of *Hha*I) and mixed by gentle flicking prior to incubating at 37°C for 2 hours on a thermal cycler.

The digestion was terminated by the addition of 2 μ l of loading buffer (Appendix I.8) and the products analysed by agarose gel electrophoresis on a 2% gel as previously described in Section 2.2.3.5.

2.3.4 PCR amplification of PTEN with High Fidelity Pfx Polymerase

2.3.4.1 Optimisation of PCR using Platinum Pfx Polymerase

Full length PTEN was amplified from each of the four staff cDNA samples using 0.5 μ l of the high fidelity proofreading DNA polymerase Platinum Pfx (Invitrogen), exonic primers PTEN 10b and 11a, and 3mM magnesium on the Pfx3lx PCR programme (Appendix V), to yield a fragment of 1.2 Kb. 10 μ l of each PCR product was analysed by agarose gel electrophoresis on a 1% gel

with 1µl of 1Kb molecular weight marker, as previously described. The PCR programme was modified to increase the yield of product (Appendix V).

2.3.4.2 Scaled-up PCR of PTEN

Four PCR reactions were performed simultaneously using the Pfx3lx2 PCR programme and conditions previously described in Section 2.3.4.1. They were pooled and a 10µl aliquot was analysed on a 1% agarose gel.

2.3.5 Preparation of the PTEN fragment for ligation

2.3.5.1 Gel purification of the PTEN fragment

Full length PTEN PCR product was separated by 1% agarose gel electrophoresis and the product band carefully excised from the gel with a sterile scalpel. DNA was purified from the agarose slices using two columns from a Qiagen Gel Extraction Kit, according to the manufacturers' instructions. Sterile water (30µl) was used to elute the DNA from each column, and the eluates were pooled.

2.3.5.2 Small-Scale Digestion of the PTEN PCR Product with Restriction Endonucleases *Eco*RI and *Sal*I

Restriction digestion was performed by mixing 28µl of gel purified PCR product with 4µl of 10x *Eco*RI buffer, 4µl of 1µg/ml BSA and 2µl each of *Eco*RI and *Sal*I in a total volume of 40µl. The digest was incubated at 37°C for 2 hours.

2.3.5.3 Purification of the *Eco* RI-SaI I digested PTEN Fragment and assessment of yield

The digested PTEN fragment was purified directly from solution using a Qiagen Gel Extraction Kit, according to the manufacturers' instructions for the clean-up of enzymatic reactions. 30µl of sterile water was used to elute the purified DNA from the spin column, and the eluate stored at -20°C. Yield was assessed as described in 2.3.7.3.

2.3.6 Transformation of *E.coli* with CMV-pEGFP-N1

2.3.6.1 Preparation of chemically competent JA221 cells by calcium chloride method

Aseptic technique was used for all bacterial manipulations, using a protocol modified from Maniatis, Sambrook & Fritsch, (1989). A 1ml aliquot of JA221 cells stored as a 50% glycerol stock at -80°C was thawed on ice. The 1ml of cells was inoculated into 100ml of LB in a 1L sterile flask and incubated at 37°C for 16 hours with shaking at 250 rpm. The following morning 10ml of the saturated culture was diluted into 90ml of LB and grown at 37°C and 250 rpm for approximately 2 hours, until an absorbance (A^{650}) of 0.5-0.6 was reached.

The culture was then transferred into sterile pre-chilled 50ml Falcon tubes and incubated on ice for 10 minutes to inhibit further growth. The cells were centrifuged at 3,500 rpm at 4°C for 5 minutes in a pre-chilled Sorvall Super T21 centrifuge and the medium carefully removed. The cell pellets were

resuspended in 10ml of ice-cold 0.1M MgCl_2 , incubated on ice for 5 minutes, and then centrifuged at 3,500 rpm at 4°C for 5 minutes.

Following careful aspiration of the supernatant, the cell pellets were gently resuspended in 1.5ml of ice-cold 0.1M CaCl_2 and held on ice in preparation for transformation. The cells were treated very carefully at this stage, as the cell membranes are highly permeable and easily ruptured if too much shear force is applied to the tube.

2.3.6.2 Transformation of Competent JA221 with CMV-pEGFP-N1

Using the competent JA221 described above, a 200 μl aliquot of cells was added to a sterile thin-walled polypropylene culture tube for transformation using pre-chilled pipette tips, based on a protocol modified from Maniatis, Sambrook & Fritsch, (1989). 1 μl of CMV-pEGFP-N1 was added to the cells and mixed by swirling gently with the pipette tip; the cells were then incubated on ice for 30 minutes to allow the plasmid DNA to bind to the bacterial cell wall.

To induce bacterial uptake of the DNA, the cells were heat-shocked by placing the tube in a water bath at exactly 42°C for 90 seconds, then transferred immediately to ice for 2 minutes. 800 μl of room-temperature SOC medium (Appendix I.4) was added to the cells, which were incubated at 37°C for 1 hour at 220 rpm to allow expression of the kanamycin resistance gene encoded by the plasmid.

2.3.6.3 Plating out of the transformants

From the transformed cells prepared in Section 2.3.6.1, a 100µl aliquot was spread onto an L-Agar plate containing 50µg/ml kanamycin (Appendix I.4) using a sterile spreader and the plate inverted and placed in an incubator at 37°C overnight. The plate was inspected for colony formation the following morning and stored inverted in a refrigerator until required.

2.3.7 Preparation of CMV-pEGFP-N1 Plasmid for Cloning

2.3.7.1 Small Scale Isolation of CMV-pEGFP-N1 DNA by Alkaline Lysis

From the plate prepared as described in Section 2.3.6.3, a single colony was picked using a sterile inoculating loop into 5ml of LB with kanamycin, (Appendix I.4), and incubated at 37°C for 16 hours at 250 rpm (Maniatis, Sambrook & Fritsch, (1989). Culture (3ml in total) was microcentrifuged in a 1.5ml Eppendorf tube at 13,000 rpm for 5 minutes, and the pellet resuspended by vortexing in 100µl of ice-cold Solution I (Appendix I.5).

Solution II (200µl, Appendix I.5) was added to the cell suspension and the tube gently inverted 5 times to lyse the cells. After incubation on ice for 3 minutes the lysate was observed to become clear, indicating complete lysis, and 150µl of ice-cold Solution III (Appendix I.5) was then added.

The tube was inverted twice and incubated on ice for 5 mins, prior to microcentrifugation at 13,000 rpm for 5 minutes at 4°C. 400µl of supernatant was transferred to a fresh sterile tube, an equal volume of phenol:chloroform:isoamyl alcohol added and the solution vortexed for 10 seconds. The phases were separated by centrifugation at 13,000 rpm for 2 minutes and 350µl of the supernatant transferred to a fresh sterile tube. To

precipitate the plasmid DNA 700µl of 100% ice-cold ethanol was added to the tube, mixed by inversion and incubated at -20°C for 20 minutes.

The DNA was pelleted by centrifugation at 13,000 rpm for 5 minutes at 4°C and washed with 1 ml of ice-cold 70% ethanol. A final centrifugation at 13,000rpm for 10 minutes at 4°C was used to bring the pellet back to the bottom of the tube and the ethanol carefully aspirated.

The pellet was air-dried on the bench for approximately 2 hours until the DNA appeared transparent and dry, then resuspended in 40µl of 5mg/ml RNase A solution. Presence of the plasmid was confirmed by 1% agarose gel electrophoresis using 1µl of stock plasmid and a 1Kb molecular weight marker for a size comparison and to give an estimation of yield. Plasmid DNA was stored at -20°C until required.

2.3.7.2 Small-Scale Digestion of CMV-pEGFP-N1 with restriction endonucleases *EcoRI* and *SaI*

Double digest of the plasmid was performed essentially as described in Section 2.3.5.2, using 5µl of plasmid DNA (extracted as described in Section 2.3.7.1) in a 20µl digest with 2µl of *EcoRI* (40U) and *SaI* (40U).

2.3.7.3 Gel Extraction of the *EcoRI-SaI* Linearised Plasmid

To remove the unwanted small fragment generated during the restriction digest, the digest was fractionated on a 1% agarose gel and the 4.7Kb band corresponding to linearised vector carefully excised using a sterile scalpel. The DNA was extracted using a Qiagen Gel Extraction Kit according to the manufacturers' instructions into 30µl of sterile water. Yield was assessed by

analysing 5µl of the purified digested vector and 5µl of purified digested PTEN on a 1% agarose gel. The purified plasmid was stored at -20°C until required.

2.3.8 Ligation

After assessing the yield of the double-digested PTEN fragment and plasmid, three ligation reactions were carried out at DNA molar ratios of approximately 1:1, 1:3 and 1:5 of vector : insert. This ensured that one of the reactions would contain the optimal amounts of each digested DNA fragment for efficient ligation. Ligations were performed by adding to the appropriate volumes of DNA 2.5µl of ligase buffer and 1µl of T4 DNA Ligase (NEB), in a total volume of 25µl. The reactions were incubated at 10°C for 16 hours on the block of a thermal cycler according to the instructions provided with the ligase.. A control ligation was also performed using 1µl of 100bp molecular weight marker under the same conditions, or at room temperature. To determine whether ligation had occurred, 10 µl of the three ligations and control reaction were subjected to 1% agarose gel analysis.

2.3.9 Introduction of Recombinant Plasmid DNA into E.coli cells

2.3.9.1 Transformation of Chemically Competent DH5α

Chemically competent DH5α (Invitrogen) were transformed with 5µl of each ligation reaction (Section 2.3.8), according to the manufacturers' recommendations, essentially as described in 2.3.6.2.

2.3.9.2 Plating Out and Analysis of the Transformants

LB-Agar plates containing 50µg/ml Kanamycin were prepared as described in Appendix I.4. Volumes of 20 or 50µl of each of the three transformation reactions were pipetted onto an agar plate and spread over the surface using a sterile spreader. A control plate was included which contained no antibiotic, onto which 20µl of DH5α was spread from the remainder of the stock competent cells. The plates (8 in total) were incubated at 37°C for 16 hours to allow colony formation. The number of colonies on each plate was noted by counting.

2.3.9.3 Confirmation of the presence of full length PTEN in the recombinant plasmids

Two medium-sized colonies were selected from each plate and inoculated into 5 ml of LB with 50 µg/ml kanamycin using sterile toothpicks, and grown for 16 hours at 37°C with shaking at 250 rpm. Each culture, (3ml), was used to isolate plasmid DNA by the alkaline lysis method as described in Section 2.3.7.1; to the remaining 2ml of culture an equal volume of sterile glycerol was added, shaken, and frozen at -70°C.

The extracted plasmid DNA was analysed by running 1µl of each plasmid preparation against 0.5µl of uncut pEGFP on a 1% agarose gel. A 1µl aliquot of each plasmid was then double-digested with 1µl each of *EcoRI*, *SaII* and BSA in a total volume of 20µl as described previously. 10µl of each digest were analyzed by 1% agarose gel electrophoresis against 0.5µl of undigested pEGFP and 1µl of double-digested pEGFP.

2.3.10 Preparation of pRC-2 (clone 6A) plasmid DNA for Sequencing

Upon careful analysis of the results of gel electrophoresis of the double-digested plasmid samples, the clone 6A was selected and named pRC-2. To propagate the clone, the pRC-2 glycerol stock was thawed and 20µl spread onto an L-Agar/Kan selective plate, as described in Section 2.3.9.2. After 16 hours of incubation at 37°C a single colony was picked for overnight growth in selective LB as described in 1.7.3. From this preparation a stab culture was taken by dipping a sterile inoculating loop into the culture and 'stabbing' it into a selective L-agar tube (Appendix I.4). The tube was incubated at 37°C overnight then packaged on ice and sent for sequencing to Lark Technologies.

2.3.11 Subcellular Localisation and Distribution of PTEN

2.3.11.1 Preparation of Ultrapure Supercoiled Plasmid

To improve the transfection rate of pRC-2 into all the cell lines, the plasmid was prepared by an alternative technique to the one described in Section 2.3.7.1. Fresh LB selective plate was streaked with pRC-2 glycerol stock. An overnight culture was grown from a single colony picked from the plate and the plasmid isolated using a GenElute Plasmid Miniprep Kit, according to the manufacturer's instructions. Plasmid DNA was analysed by agarose gel electrophoresis, the concentration measured spectrophotometrically at 260nm and a stock solution of 1µg/µl prepared.

2.3.11.2 Transfection of cell lines with pRC-2 by Lipid-Mediated Transfer

Initial transfections were performed on the COS-7 and HEC-1B cell lines using the pRC-2 plasmid at a stock concentration of 1µg/µl. Cells lines were seeded on 13mm round coverslips placed in the wells of 24 well culture plates and grown to 50% confluence 24 hours prior to transfection with 0.6, 0.7, 0.8, 0.9 or 1.0µg of plasmid, as recommended by the manufacturers of the Lipofectamine reagents (Invitrogen).

Two commercial transfection reagents were tested; Lipofectamine and Lipofectamine 2000 (LF2K), using 2µl of each reagent combined with each concentration of plasmid. These cationic lipid reagents bind to DNA and permit transfer across the cell membrane, and are commonly used in place of electroporation or calcium phosphate methods (Maniatis, Sambrook & Fritsch, 1989). The lipid reagents were used to transfect the cells according to the manufacturer's instructions and these cells were then maintained in culture for 24 hours post-transfection to allow expression of the fusion protein.

The cells were washed twice with PBS, fixed in 200µl of ice-cold methanol for 10 minutes (Beppu, et al, 1994), and the nuclei stained with DAPI (1µg/ml) for 5 minutes. The stain was washed off twice with PBS and the coverslips mounted on microscope slides using a small drop of Fluoromount G aqueous mountant.

Slides were analysed by manual fluorescence microscopy using a U-V filter for DAPI and FITC filter for EGFP. The microscope used was an Olympus BX60 microscope with a JVC 3-CCD digital camera and Image Grabber PC

V1.2 (Neotech Ltd,1995) software. Transfection efficiency using Lipofectamine and LF2K was recorded for each cell line.

2.3.12 Optimisation of Transfection

Adherence of the cells during fixing was improved by seeding onto coverslips that had been treated with polylysine. Prior to adding the cells, the coverslips were submerged in 200 μ l of a 5 μ g/ml solution of polylysine (Sigma) in sterile PBS for 20 minutes, according to the manufacturers' recommendations. The polylysine was aspirated, allowed to air dry for a few minutes and the cells pipetted into the wells of the culture plate. The experiment described above in 2.3.1.2 was repeated using polylysine-treated coverslips and HEC-1B and Ishikawa cell lines. Conditions resulting in the highest transfection rate and expression of EGFP were noted for each cell line.

2.3.13 Stimulation of Transfected Cell lines

The effect of TGF- β 1 and estrogen on the localization of pRC-2 was investigated in the HEC-1B and Ishikawa cell lines. The cell lines were transfected with 0.8-1.0 μ g of pRC-2 using 2 μ l Lipofectamine prior to treatment with the relevant compound.

2.3.13.1 Oestrogen

Transfected cells were stimulated with 10 or 100nM β -Oestradiol (Appendix I.6) for various periods of time, based on work by Campbell, et al (2001). The

complete growth medium was replaced with reduced-serum medium (Appendix I.1) and the appropriate volume of hormone from the stock solution. HEC-1B cells were stimulated for 30 minutes and 1, 2, 6, or 24 hours. Ishikawa cells were stimulated for 24 or 48 hours using reduced-serum medium with and without phenol red, to ascertain whether this molecule (which has structural similarity to oestrogen) was affecting the results. After each time point the coverslips were washed, fixed, DAPI stained and mounted for microscopic analysis as described in Section 2.3.11.2.

2.3.13.2 TGF- β 1

Transfected cells were stimulated with 2 or 5ng/ml of TGF- β 1 (Appendix I.6) for 30 minutes, 1, 2 and 6 hours. After transfection the complete growth medium was replaced with reduced-serum medium (Appendix I.1) and the appropriate volume of cytokine from the stock solution. When stimulation was complete the coverslips were prepared for microscopic analysis as previously described.

2.3.14 Localisation of pRC-2 to Subcellular Compartments

To determine the sequestration of pRC-2 to subcellular compartments, transfected HEC-1B and Ishikawa and were used in double-labelling experiments. Monoclonal antibodies to the ER protein Colligin (Stressgen), PTEN (BD) and β -Actin (Sigma), were used according to the manufacturers' guidelines. A polyclonal antibody against the golgi protein Mannosidase II

The supernatant was carefully removed and the pellet resuspended in 20 ml of Buffer A, prior to centrifugation at 3000 rpm for 15 minutes at room temperature. The supernatant was again discarded and the pellet resuspended in 1 ml of Buffer B (Appendix I.7), and transferred to a sterile 1.5 ml Eppendorf tube.

5M Sodium perchlorate (300µl) was added to the samples, which were mixed end-over-end for 10 minutes at room temperature followed by microcentrifugation at 14,000 rpm for 10 minutes at 4°C in a Heraeus Microcentaur. To fresh Eppendorf tubes 600 µl of supernatant was transferred and 700 µl of ice-cold chloroform added. The samples were mixed end-over-end for 3 minutes at 4°C and then centrifuged at 14,000 rpm for 10 minutes at 4°C.

The top layer of each sample was carefully transferred to a clean tube and two volumes of ice-cold ethanol added, with gentle mixing. A final centrifugation at 14,000 rpm for 15 minutes at 4°C was performed, and the supernatant aspirated from resulting DNA pellets, which were air dried for 10 minutes. The dried pellets were resuspended in 200-500 µl of sterile distilled water and stored at -70°C.

2.4.1.2 Extraction of Patient DNA from archival material

DNA from archival material was extracted and made available for my use by kind permission of Dr Ken Feeley, University of Sheffield Medical School. The extractions were performed by a member of Dr Feeleys technical staff.

was kindly donated by Prof David Parkinson, and used according to his recommendations. Cell lines transfected as described previously were fixed in methanol, blocked for 1 hour with normal goat serum (NGS, Appendix II.6) and incubated with α -Colligin (1:200), α -Mannosidase II (1:70), α -PTEN (1:200) and α - β -Actin (1:20,000) in NGS for 1 hour. The cells were washed twice with PBS and incubated with secondary α - mouse / rabbit Alexafluor 594 (1:200) in NGS for 45 minutes according to the enclosed instructions; the cells were then washed, DAPI stained and mounted.

2.4 MUTATIONAL ANALYSIS OF PTEN EXONS 3 & 5 IN ENDOMETRIAL DNA SAMPLES BY SINGLE STRAND CONFORMATION POLYMORPHISM ANALYSIS (SSCP)

2.4.1 Preparation of DNA Samples for SSCP Analysis

2.4.1.1 Extraction of DNA from whole human blood

Fresh human venous blood was collected from staff members into vacutubes containing EDTA and the tubes marked anonymously for processing using a method modified from Maniatis, Sambrook & Fritsch, (1989). Each blood sample (10-20ml) was transferred to sterile universal tubes and Buffer A (Appendix I.7) added to make a total volume of 50 ml. The samples were mixed end-over-end for 4 minutes at room temperature, followed by centrifugation at 3000 rpm for 15 minutes at room temperature in a Sorvall RT7 Plus centrifuge.

2.4.2 PCR of Patient & Control DNA for SSCP Analysis

PCR was carried out on patient DNA samples extracted from paraffin embedded archival tissue, and on control DNA prepared as described in Section 2.4.1.1. Oligonucleotide primers designed to amplify PTEN Exons 5 and 8, (Risinger et al, 1997), in two overlapping fragments of approximately 200bp in length for each exon were used. Primers 5A/5B and 5C/5D (Section 2.1.3) produced PCR products of 137bp and 186bp respectively, and 8A/8B and 8C/8D produced PCR products of 122bp and 245bp respectively.

PCR was performed using 1-5 μ l of patient DNA, 3mM magnesium chloride, 0.2 mM dNTPs, 0.5 μ l of RedHot Taq polymerase and 50pM of forward and reverse primer in 1 x reaction buffer, in a total volume of 25 μ l. Reactions were carried out using the PTEN I programme for all primer pairs 5C/D and 8A/B or the RIS2-2 programme for primers 5A/B (Appendix V). The PCR products (10 μ l) were analysed by agarose gel electrophoresis.

2.4.3 Preparation of Control PCR products for sequencing

To verify that the PCR products amplified from the control DNA were wild-type, and therefore suitable for use as SSCP controls, PCR fragments for each primer pair were sequenced following the extraction from 2% agarose gels using a Qiagen Gel Extraction Kit. The purified fragments were vacuum desiccated and sent to Lark Technologies where they were automatically sequenced using the respective PCR primers.

2.4.4 SSCP analysis

2.4.4.1 Large gel format, Hoefer 18 x 24cm

SSCP was performed essentially as described by Rhei, et al (1997) and Aveyard, et al (1999). A large format gel was used to enable the samples to be electrophoresed slowly overnight, (to minimize heat generation), and to give maximum separation of bands. The SSCP plates were cleaned thoroughly with ethanol and assembled using 1mm spacers. Gels were prepared at concentrations of either 0.5 x MDE, for exon sections 5CD and 8AB, or 0.6 x MDE for exon sections 5AB and 8CD all with 5% or 10% glycerol (Appendix I.9) and cast between the assembled glass plates.

Teflon combs (15 wells) were inserted into the gels, which were allowed to polymerize at room temperature for 3 hours. The polymerized gels were then chilled to 4°C for 2 hours, the combs were removed and the wells rinsed thoroughly with chilled 0.6 x TBE running buffer (Appendix I.9). The gels were then clamped into the gel tank and submerged in running buffer.

PCR products were prepared for SSCP analysis by mixing 2-4µl of sample with an equal volume of formamide buffer (Appendix I.9) and sterile water to a total volume of 10µl. Samples were then heated at 95°C for 10 minutes on a thermal cycler before being transferred to ice for 2 minutes. 10µl of each sample were loaded into the wells of the gel using gel loading tips; 0.5µl of molecular weight marker was also included in one well of each gel. The samples were subjected to electrophoresis at 18W constant power for 16 hours with an ambient temperature of 4°C.

2.4.4.2 Silver staining

Upon completion of electrophoresis the plates were removed from the tank and the gels developed initially using a silver staining protocol based on that of Merril, (1990). Briefly, the gels were fixed for 10 minutes, incubated with a silver solution for 15 minutes and washed with distilled water, prior to developing for 15 minutes. The gels were washed again and then a final fixer solution applied for 10 minutes (Appendix I.9).

To improve the sensitivity of detection a PlusOne DNA Silver Staining kit was later used for all gels, according to the manufacturer's instructions. Stained bands were visualized on a visible light box and photographed using a Kodak DC120 digital camera before being sealed in BioDesign gel wrap and allowed to dehydrate at room temperature for 24 hours. Bands observed for each patient sample were compared to those corresponding to the control DNA samples on each gel, and samples exhibiting bandshifts or extra bands were subjected to a repeated gel run.

For samples with confirmed bandshifts, the remaining PCR product was packaged on ice with aliquots of the relevant primer pairs and sent to Lark Technologies for sequencing.

CHAPTER 3

RESULTS

3.1 ANALYSIS OF EXPRESSION OF PTEN AND SELECTED GENES IN HEC-1B AND ISHIKAWA CELL LINES STIMULATED WITH TGF- β 1

The aim of this section was to evaluate the response of PTEN to stimulation by TGF- β 1 in endometrial cell lines. Several studies of this nature have previously been published describing the response of PTEN to TGF- β 1 in other cell lines, including keratinocytic and myeloid, but the data from these papers is conflicting and appears to be cell-line specific. Compounds which up-regulate PTEN expression may represent a useful therapeutic strategy in the non-surgical treatment of tumours.

To assess any biologically significant changes in PTEN expression, both mRNA and protein levels were studied in two epithelial cell lines of endometrial adenocarcinoma origin. The HEC-1B cell line, representing a moderately differentiated grade II carcinoma (Crescenzi & Palumbo, 2001), possesses wild-type PTEN (Yaginuma, et al 2000) and expresses the protein (Figure 10.1). Conversely, the Ishikawa cell line represents a well differentiated carcinoma, but has a frameshift deletion in exon 8 of PTEN (codon 289 deletion of A) which codes for a rapidly degraded protein truncated at codon 290 (Yaginuma, et al 2000).

For experimental control purposes RNA and protein expression of the housekeeping genes GAPDH and β -Actin were studied. Housekeeping genes, including those indicated, are constitutively expressed and necessary for cell survival, with highly stable expression levels that are unlikely to change under experimental conditions. The TGF- β 1 responsive gene PAI-1

was also analysed, as levels of this gene have been widely reported to increase during TGF- β 1 stimulation in many cell lines (Keeton, et al 1991, Fujimoto, et al 1996 & Jiang, et al 2003). mRNA expression of the pro-apoptotic protein Bcl-2 in HEC-1B was also analysed by RT-PCR, as this protein has been reported to be down-regulated in this cell line (Crescenzi, et al 2000). Levels of phospho-PTEN protein were also analysed using an antibody specific for phosphorylated Ser380, as phosphorylation of the PTEN tail influences stability, degradation and activity of the protein.

Stimulation with cytokine was performed in the presence of either 10% or 1% foetal calf serum, which contains varying amounts of TGF- β 1. The 10-fold reduction in serum concentration was therefore used to discount any effects of cytokine in the growth medium. Cellular morphology, density, and proliferation were also studied to further elucidate any correlation between TGF- β 1 stimulation and PTEN expression.

3.1.1 Analysis of mRNA expression

Cells were grown under serum-starved and non-starved conditions in order to assess any differences in response to TGF- β 1 under different growth conditions. The cells were then treated with either 2ng/ml or 5ng/ml TGF- β 1, RNA was extracted and quantitation of the yield and purity of RNA from each sample was performed. These procedures are detailed in Chapter 2, Sections 2.2.1 to 2.2.3. The results shown in Appendix VII indicated a good yield of RNA, the quality of which would be shown by electrophoresis. All samples were diluted to 1 μ g/ μ l prior to use in PCR.

Semi-quantitative analysis of mRNA for PTEN, PAI-1 and GAPDH

RT-PCR was performed as described in Chapter 2 Section 2.2.3 and the products separated by agarose gel electrophoresis (Chapter 2, Section 2.2.3.5). Results are displayed in Figures 10 to 14 as a time course (4 to 24 hours) with TGF- β 1 concentrations of 2ng/ml in red, 5ng/ml in blue and the control (no TGF- β 1) in white. A Yellow line indicates PCR control minus cDNA template and duplicate sets of data are labelled A and B. A 100bp ladder was included on each gel. Densitometric analysis of PCR products was performed as described in Chapter 2 and shown in Tables 3 to 7. Results below the detection limit of the software are indicated by the notation “nd” (no data). Using Microsoft Excel, the overall results of PTEN mRNA expression for each experiment were expressed graphically by plotting the mean mass of DNA for each set of data points (Graphs 1 to 3). Error bars were added after calculating the standard error of the mean values.

Results for HEC-1B serum non-starved electrophoresis are shown in Figures 10.1 to 10.4, and densitometric analysis is shown in Table 3 and Graph 1. This experiment was performed once, in duplicate. Results for HEC-1B serum starved electrophoresis experiments 1 and 2 are shown in Figures 11.1 to 11.4 and Figures 12.1 to 12.4, respectively, and densitometric analysis is shown in Table 4 and Table 5 respectively. Overall results for both independent experiments (performed in duplicate) are illustrated in Graph 2.

Results for Ishikawa serum non-starved electrophoresis are shown in Figures 13.1 to 14.4, and densitometric analysis is shown in Tables 6 and 7. This experiment was performed once in duplicate, and cDNA synthesis performed twice, in duplicate. Results for both experiments are illustrated in Graph 3.

Serum non-starved HEC-1B

Ong
4 8 24

I

A

B

Fig 10.1 PTEN expression in non-starved HEC-1B with duplicates A and B.

No TGF- π control shown in white, 2ng/ml TGF-p1 in red, 5ng/ml TGF- π in blue over 4, 8 & 24 hours. PCR control (no DNA) is a yellow dash.

A

B

Fig 10.2 GAPDH expression in non-starved HEC-1B with duplicates A and B.

No TGF-p1 control shown in white, 2ng/ml TGF- π in red, 5ng/ml TGF- π in blue over 4, 8 & 24 hours. PCR control (no DNA) is a yellow dash.

A

B

Fig 10.3 PAI-1 expression in non-starved HEC-1B with duplicates A and B.

No TGF-p1 control shown in white, 2ng/ml TGF-pi in red, 5ng/ml TGF-pi in blue over 4, 8 & 24 hours. PCR control (no DNA) is a yellow dash.

Ong/4

Fig 10.4 -RT controls for PTEN expression in non-starved HEC-1B, all samples and duplicates A/B. PCR control shown as yellow dash.

Fig 10.5 Expression of 1: PTEN and 2: TPIP with PCR control (yellow dash) in HEC-1B.

Serum non-starved HEC-1B stimulated with TGF- β 1

This experiment was performed once, with duplicate flasks A and B from which cDNA was prepared for RT-PCR using primers for PTEN, PAI-1 and GAPDH. The analysis of RT-PCR products shown in Figure 11.1 show a subtle time and dose-dependant increase in PTEN mRNA expression in samples stimulated with TGF- β 1 when compared to the controls. The response of PAI-1 in Figure 10.3 indicated a strong time and dose-dependant increase in expression (approximately four-fold) compared to the unstimulated controls, in a manner concurrent with published data (Lee, et al 1999).

2ng/ml TGF- β 1

From Figure 10.1 it was seen that under stimulation with 2ng/ml of cytokine, no increase in PTEN expression was detected at 4 hours. An average increase in expression of ~1.5-times was observed at 8 hours but little change at 24 hours was observed compared to the controls.

Figure 10.3 shows PAI-1 mRNA expression in response to cytokine stimulation. At 4 hours, an increase of ~4-times compared to the controls was observed. At 8 and 24 hours an increase in PAI-1 expression of ~3.5-times and 3-times respectively was detected in comparison to the controls.

5ng/ml TGF- β 1

Samples stimulated with 5ng/ml of TGF- β 1 exhibited an increase in PTEN expression at 4 hours of ~1.4 times. At 8 hours the average increase in

expression was ~2-times that of the controls and at 24 hours a small increase of ~1.2-times was observed.

Figure 10.3 shows PAI-1 mRNA expression in response to cytokine stimulation. At 4 hours, an increase of ~5-times compared to the controls was observed. At 8 and 24 hours an increase in PAI-1 expression of ~4-times and 3-times respectively was detected in comparison to the controls.

Expression levels of GAPDH shown in Figure 10.2 remained constant in all samples, indicating that TGF- β 1 had no discernable effect on this housekeeping gene.

Figure 10.4 shows RT-PCR of reverse transcription controls using the PTEN primers 2a and 3. All samples are free of PCR product and therefore the cDNA samples prepared were free of DNA contamination. The results observed in Figures 10.1 to 10.3 were also shown by densitometric analysis of band intensities in Table 3.

The additional PCR shown in Figure 10.5 was performed using 4 hour control cDNA and primers for PTEN (2a & 3) and TPIP (TPTE and PTEN homologous inositol lipid phosphatase). Sequences of the primers are listed in Chapter 2, Section 2.1.3. This PCR was included to demonstrate that amplification of TPIP, a PTEN homologue, had not occurred and therefore all such PCR reactions were specific for PTEN only.

Expression of Bcl-2 mRNA was undetectable in the 1:4 dilutions of cDNA used to perform RT-PCR. Feint bands were visible in all samples when undiluted cDNA was used, indicating that this cell line expressed very little Bcl-2 mRNA, and stimulation with TGF- β 1 did not noticeably alter expression levels. The results are not shown due to the feint bands being very difficult to

photograph. Very low expression of Bcl-2 protein has previously been reported in the HEC-1B cell line, and is indicative of malignant change from hyperplasia to carcinoma (Crescenzi & Palumbo, 2001).

Summary of Results

A modest up-regulation of PTEN mRNA was observed in cells stimulated with TGF- β 1 compared to the controls. The increase in transcripts was more strongly observed in cells stimulated with 5ng/ml of cytokine, which was seen to be approximately double after 8 hours compared to the unstimulated controls.

| Table 3 HEC-1B Serum non-starved PCR Densitometry | | | | | |
|---|------------|------------|-----------------------------------|------------|------------|
| PTEN PCR A | | | PTEN PCR B | | |
| TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD | TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD |
| 0ng/4 | 41.15 | 161 | 0ng/4 | 59.49 | 161 |
| 8 | 114 | 187 | 8 | 123.6 | 194 |
| 24 | 199.7 | 194 | 24 | 224.4 | 220 |
| 2ng/4 | 28.31 | 91 | 2ng/4 | 45.43 | 91 |
| 8 | 169.6 | 194 | 8 | 224.4 | 224 |
| 24 | 209.6 | 210 | 24 | 253.3 | 224 |
| 5ng/4 | 63.45 | 142 | 5ng/4 | 74.3 | 139 |
| 8 | 194.8 | 207 | 8 | 254.6 | 227 |
| 24 | 237.5 | 210 | 24 | 280.2 | 251 |
| GAPDH PCR A | | | GAPDH PCR B | | |
| TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD | TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD |
| 0ng/4 | 332.11 | 252 | 0ng/4 | 321.9 | 251 |
| 8 | 330.31 | 252 | 8 | 320.64 | 253 |
| 24 | 332.13 | 252 | 24 | 321.71 | 253 |
| 2ng/4 | 330.4 | 251 | 2ng/4 | 313.2 | 250 |
| 8 | 332 | 253 | 8 | 319.81 | 253 |
| 24 | 334.2 | 254 | 24 | 320.21 | 254 |
| 5ng/4 | 331.16 | 253 | 5ng/4 | 320.3 | 252 |
| 8 | 332.11 | 254 | 8 | 320.6 | 254 |
| 24 | 333.5 | 254 | 24 | 321 | 252 |
| PAI-1 PCR A | | | PAI-1 PCR B | | |
| TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD | TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD |
| 0ng/4 | 40.93 | 89 | 0ng/4 | 41.45 | 91 |
| 8 | 70.12 | 117 | 8 | 70.55 | 117 |
| 24 | 110.67 | 117 | 24 | 114.4 | 118 |
| 2ng/4 | 176.3 | 218 | 2ng/4 | 179.4 | 218 |
| 8 | 261.5 | 245 | 8 | 261.3 | 245 |
| 24 | 295.1 | 254 | 24 | 293.2 | 252 |
| 5ng/4 | 200 | 220 | 5ng/4 | 196.6 | 220 |
| 8 | 285.64 | 254 | 8 | 287.3 | 238 |
| 24 | 294.4 | 254 | 24 | 296.8 | 238 |

Serum starved HEC-1B, Experiment 1

A

B

Fig 11.1 PTEN expression in serum-starved HEC-1B with duplicates A and B. No TGF- β 1 control shown in white, 2ng/ml TGF-p1 in red, 5ng/ml TGF-p1 in blue over 4, 8 & 24 hours. PCR control (no DNA) is a yellow dash.

Ong
_J0%4 a 24

A

B

Figure 11.2 PAI-1 expression in serum-starved FIEC-1B with duplicates A and B. No TGF-P1 control shown in white, 2ng/ml TGF-pi in red, 5ng/ml TGF-pi in blue over 4, 8 & 24 hours. PCR control (no DNA) is a yellow dash.

**Fig 11.3 GAPDH expression in serum-starved HEC-1B with
duplicates A and B. All samples from TGF-(31 (white) to
5ng/ml TGF-(31 (red) and duplicates A/B. PCR control shown
as yellow dash**

**Fig 11.4 -RT controls for PTEN expression in serum starved
HEC-1B. All samples from TGF-(31 (white) to 5ng/ml TGF-(31
(red) and duplicates A/B. PCR control shown as yellow dash.**

Serum-starved HEC-1B stimulated with TGF- β 1

This experiment was performed twice, with duplicate flasks A and B from which cDNA was prepared for RT-PCR using primers for PTEN, PAI-1 and GAPDH. The two experiments were performed independently and were donated experiments 1 and 2 respectively.

Experiment 1

The analysis of RT-PCR products shown in Figure 11.1 show a subtle time and dose-dependant increase in PTEN mRNA expression in samples stimulated with TGF- β 1 when compared to the controls, with a slightly different response pattern to that observed in the non-starved experiments. Reduction of serum initiated a 2-fold increase in expression, as observed between the 10% serum controls and 4 hour serum-starved controls. Modulation of PTEN expression during serum starvation has been reported previously in neuronal cells (Kyrylenko, et al 1999). The response of PAI-1 in Figure 11.3 indicated a strong time and dose-dependant increase in expression (approximately three-fold) compared to the unstimulated controls.

2ng/ml TGF- β 1

From Figure 11.1 it was seen that under stimulation with 2ng/ml of cytokine, no increase in PTEN expression was detected at 4 hours. An average increase in expression of ~2-times was observed at 8 hours and maintained over 24 hours compared to the controls.

Figure 11.2 shows PAI-1 mRNA expression in response to cytokine stimulation. At 4 hours, negligible increase of expression compared to the

controls was observed. At 8 and 24 hours an increase in PAI-1 expression of ~2-times and 3-times respectively was detected in comparison to the controls.

5ng/ml TGF- β 1

Samples stimulated with 5ng/ml of TGF- β 1 exhibited an increase in PTEN expression at 4 hours of ~2-times. At 8 hours the average increase in expression was ~2-times, which was again maintained over the 24 hour period when compared to the controls.

Figure 11.2 shows PAI-1 mRNA expression. At 4 hours, very little increase of expression compared to the controls was observed. At 8 and 24 hours an increase in PAI-1 expression of ~2-times and 3-times respectively was detected in comparison to the controls.

Expression levels of GAPDH shown in Figure 11.3 remained constant in all samples, indicating that TGF- β 1 had no detectable effect on this housekeeping gene.

Again, Bcl-2 levels were undetectable in diluted cDNA samples but faint bands were detected using undiluted cDNA as observed for serum non-starved HEC-1B. TGF- β 1 did not appear to alter expression of Bcl-2 mRNA from the slight bands seen in the controls (data not shown).

Figure 11.4 shows RT-PCR of reverse transcription controls using the PTEN primers 2a and 3. All samples are free of PCR product and therefore the cDNA samples prepared were free of DNA contamination. The results observed in Figures 11.1 to 11.3 were also shown by densitometric analysis of band intensities in Table 4.

| Table 4 HEC-1B Serum-Starved Experiment 1 PCR Densitometry | | | | | |
|--|------------|------------|-----------------------------------|------------|---------|
| PTEN PCR A | | | PTEN PCR B | | |
| TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD | TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD |
| 10% | 82.76 | 175 | 10% | 63.6 | 128 |
| 0ng/4 | 124.4 | 205 | 0ng/4 | 122.63 | 206 |
| 8 | 115.6 | 208 | 8 | 116 | 214 |
| 24 | 69.99 | 156 | 24 | 68.91 | 158 |
| 2ng/4 | 141.5 | 192 | 2ng/4 | 143.2 | 205 |
| 8 | 264.7 | 250 | 8 | 264 | 250 |
| 24 | 130.7 | 196 | 24 | 126.4 | 193 |
| 5ng/4 | 149 | 215 | 5ng/4 | 149 | 214 |
| 8 | 254.7 | 247 | 8 | 255.1 | 246 |
| 24 | 132 | 212 | 24 | 130.2 | 202 |
| GAPDH PCR A | | | GAPDH PCR B | | |
| TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD | TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD |
| 10% | 332.4 | 255 | 10% | 330.2 | 247 |
| 4 | 333.1 | 254 | 4 | 331.11 | 252 |
| 8 | 333 | 255 | 8 | 331 | 252 |
| 24 | 331.64 | 254 | 24 | 333.7 | 252 |
| 2ng/4 | 331.11 | 254 | 2ng/4 | 329.6 | 252 |
| 8 | 330.16 | 254 | 8 | 334.3 | 251 |
| 24 | 330.9 | 255 | 24 | 332 | 251 |
| 5ng/4 | 330 | 253 | 5ng/4 | 321.9 | 250 |
| 8 | 332 | 253 | 8 | 332 | 253 |
| 24 | 334.2 | 255 | 24 | 331.8 | 254 |
| PAI-1 PCR A | | | PAI-1 PCR B | | |
| TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD | TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD |
| 10% | 47.3 | 92 | 10% | 60 | 146 |
| 0ng/4 | 54 | 102 | 0ng/4 | 61.4 | 143 |
| 8 | 56.4 | 122 | 8 | 61.1 | 147 |
| 24 | 40.2 | 87 | 24 | 47.5 | 96 |
| 2ng/4 | 60.8 | 147 | 2ng/4 | 76 | 151 |
| 8 | 89.6 | 174 | 8 | 113.3 | 196 |
| 24 | 110.3 | 193 | 24 | 176 | 219 |
| 5ng/4 | 61 | 149 | 5ng/4 | 64 | 155 |
| 8 | 92.5 | 188 | 8 | 120.7 | 204 |
| 24 | 121 | 201 | 24 | 200.6 | 241 |

Serum starved HEC-1B, Experiment 2

A

B

Fig 12.1 Expression of PTEN in serum-starved HEC-1B with duplicates A and B. No TGF- β 31 control shown in white, 2ng/ml TGF-pi in red, 5ng/ml TGF-pi in blue over 4, 8 & 24 hours. PCR control (no DNA) is a yellow dash.

A

B

Figure 12.2 Expression of GAPDFI in serum-starved FIEC-1B with duplicates A and B. No TGF-pi control shown in white, 2ng/ml TGF-pi in red, 5ng/ml TGF-pi in blue over 4, 8 & 24 hours. PCR control (no DNA) is a yellow dash.

Ong
: 10% 4 8 24

O n g

s m

A

B

Fig 12.3 Expression of PAI-1 in serum-starved HEC-1B with duplicates A and B. No TGF-p1 control shown in white, 2ng/ml TGF-pi in red, 5ng/ml TGF-p1 in blue over 4, 8 & 24 hours. PCR control (no DNA) is a yellow dash

Fig 12.4 -RT controls for PTEN expression in serum-starved HEC-1B with duplicates A and B in green No TGF-pi control (white), to 5ng/ml TGF-pi (red) over 4, 8 & 24 hours. PCR control (no DNA) is a yellow dash

Experiment 2

The analysis of RT-PCR products shown in Figure 12.1 again show a subtle overall increase in PTEN mRNA expression in samples stimulated with TGF- β 1, with a two-fold peak at 8 hours as observed in serum-starved Experiment 1. This increase was again maintained over 24 hours as seen in Experiment 1 (Figure 11.1), with the exception of the 5ng/ml sample at 24 hours from flask A. This sample did not show the 2-fold increase in PTEN expression and was most likely due to experimental error.

Expression levels of GAPDH shown in figure 12.2 were constant, and PAI-1 in Figure 12.3 showed the same 3-fold increase in expression as seen in Experiment 1. Results for Bcl-2 were as described for serum-starved experiment 1, with only faint bands detected in RT-PCR with undiluted cDNA and no change in expression under TGF- β 1 stimulation (data not shown). All RT-PCR controls were free of contamination as shown in Figure 12.4 and the results observed in Figures 12.1 to 12.3 were additionally shown by densitometric analysis of band intensities (Table 5).

Summary of Results for Experiments 1 & 2

A modest up-regulation of PTEN mRNA was observed in cells stimulated with TGF- β 1 compared to the controls. The increase in transcripts was more strongly observed in cells stimulated with 5ng/ml of cytokine, which was seen to be approximately double after 4 hours compared to the unstimulated controls, and was maintained over the 24 hour period.

In both serum non-starved and serum-starved HEC-1B cells treated with TGF- β 1, a modest time and dose-dependant increase in PTEN mRNA

expression was observed. This change in expression was quite similar regardless of serum levels, although serum-starvation did appear to yield a stronger induction of PTEN mRNA at 8 hours of stimulation with both concentrations of cytokine.

Notable, the response appeared to differ slightly between the controls for these experiments which were not treated with cytokine, indicating the presence of serum may alter PTEN mRNA levels. For example, PTEN expression in serum non-starved HEC-1B controls increases slightly in a linear manner over the 24 period of incubation. Cells which were serum-starved, however, demonstrated a gradual reduction in PTEN transcript levels in a linear manner over the 24 hour period. The significance of this is not clear from these results, but as a decrease in PTEN mRNA levels was observed in the controls in reduced serum medium this could be due to lower level of cytokines in the medium, including TGF- β 1, reducing overall PTEN levels.

| Table 5 HEC-1B serum-starved experiment 2 PCR densitometry | | | | | |
|--|------------|------------|------------------------------------|------------|------------|
| PTEN PCR A | | | PTEN PCR B | | |
| TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD | TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD |
| 10% | 149.7 | 200 | 10% | 139 | 191 |
| 0ng/4 | 163.8 | 204 | 0ng/4 | 164.1 | 201 |
| 8 | 133.2 | 204 | 8 | 167.5 | 203 |
| 24 | 59.28 | 157 | 24 | 167.3 | 210 |
| 2ng/4 | 70.1 | 173 | 2ng/4 | 143.7 | 209 |
| 8 | 187.4 | 252 | 8 | 176.4 | 225 |
| 24 | 149.3 | 165 | 24 | 170 | 224 |
| 5ng/4 | 147.3 | 220 | 5ng/4 | 151.8 | 207 |
| 8 | 187.6 | 257 | 8 | 188.4 | 236 |
| 24 | 57.9 | 150 | 24 | 165.2 | 205 |
| GAPDH PCR A | | | GAPDH PCR A | | |
| TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD | TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD |
| 10% | 329.3 | 249 | 10% | 334.7 | 245 |
| 0ng/4 | 329 | 241 | 0ng/4 | 332.6 | 244 |
| 8 | 329.11 | 241 | 8 | 334.4 | 245 |
| 24 | 323.21 | 241 | 24 | 333.28 | 243 |
| 2ng/4 | 330 | 247 | 2ng/4 | 329.8 | 244 |
| 8 | 330.4 | 246 | 8 | 333.71 | 246 |
| 24 | 333 | 246 | 24 | 334.67 | 247 |
| 5ng/4 | 332 | 244 | 5ng/4 | 330.5 | 241 |
| 8 | 334.2 | 245 | 8 | 333.5 | 243 |
| 24 | 332.5 | 245 | 24 | 333.2 | 245 |
| PAI-1 PCR A | | | PAI-1 PCR B | | |
| TGF- β 1 (ng) /Time (hrs) | Mass ng | Peak OD | TGF- β 1 (ng) /Time (hrs) | Mass ng | Peak OD |
| 10% | 63 | 147 | 10% | 50 | 103 |
| 0ng/4 | 64 | 149 | 0ng/4 | 51.1 | 110 |
| 8 | 66.4 | 149 | 8 | 51 | 114 |
| 24 | 65 | 134 | 24 | 53.3 | 125 |
| 2ng/4 | 74 | 153 | 2ng/4 | 74.9 | 158 |
| 8 | 115.1 | 189 | 8 | 112.4 | 195 |
| 24 | 174.6 | 217 | 24 | 147 | 210 |
| 5ng/4 | 66 | 152 | 5ng/4 | 80 | 156 |
| 8 | 117.2 | 200 | 8 | 123.6 | 202 |
| 24 | 197 | 237 | 24 | 205 | 240 |

Serum non-starved Ishikawa cells, Experiment 1

A

B

Fig 13.1 Expression of PTEN in non-starved Ishikawa with duplicates A and B. No TGF-(31 control shown in white, 2ng/ml TGF-p1 in red, 5ng/ml TGF-pi in blue over 4, 8 & 24 hours. PCR control (no DNA) is a yellow dash

A

B

Fig 13.2 Expression of GAPDH in non-starved Ishikawa with duplicates A and B. No TGF-(31 control shown in white, 2ng/ml TGF-p1 in red, 5ng/ml TGF-01 in blue over 4, 8 & 24 hours. PCR control (no DNA) is a yellow dash.

Ong
£ 4 8 24

A

B

Fig 13.3 Expression of PAI-1 in non-starved Ishikawa with duplicates A and B. No TGF-(31 control shown in white, 2ng/ml TGF-p1 in red, 5ng/ml TGF-pi in blue over 4, 8 & 24 hours. PCR control (no DNA) is a yellow dash

Ong

A

B

Fig 13.4-RT controls for PTEN expression in non-starved Ishikawa with duplicates A and B. No TGF-(31 control shown in white, 2ng/ml TGF-01 in red, 5ng/ml TGF-p1 in blue over 4, 8 & 24 hours. PCR control (no DNA) is a yellow dash

Serum non-starved Ishikawa cells stimulated with TGF- β 1

This experiment was performed once, with duplicate flasks A and B. From these duplicate flask, cDNA was prepared twice on separate occasions for RT-PCR using primers for PTEN, PAI-1 and GAPDH. The two individual batches of cDNA were designated as experiments 1 and 2 respectively.

Experiment 1

The analysis of RT-PCR products shown in figure 13.1 show a modest time and dose-dependant two-fold increase in PTEN mRNA expression in samples stimulated with TGF- β 1 at 8 hours, similar to that observed in serum non-starved HEC-1B. This cell line, which has a mutant copy of PTEN, also responded to cytokine stimulation by up-regulating expression of the TGF- β 1-responsive gene PAI-1, but the expression pattern was different from that observed for HEC-1B. In ISK cells, induction of mRNA for PAI-1 was rapid and maximal at 4 hours, declining thereafter. This pattern of expression has also been described in A594 (Keska-Oja, et al 1988) and rat mesangial cells (Jiang, et al 2003).

2ng/ml TGF- β 1

From Figure 13.1 it was seen that under stimulation with 2ng/ml of cytokine, no increase in PTEN expression was detected at 4 hours. An average increase in expression of ~2-times was observed at 8 hours and maintained over 24 hours compared to the controls.

Figure 13.3 shows PAI-1 mRNA expression in response to cytokine stimulation is essentially the reverse of that observed in HEC-1B. At 4 hours,

a ~2-fold increase in expression compared to the controls was observed. At 8 and 24 hours the levels of PAI-1 expression slowly diminished but was still detectable after 24 hours, whereas no bands were observed in the unstimulated controls.

5ng/ml TGF- β 1

Figure 13.1 shows that under stimulation with 5ng/ml of cytokine, no increase in PTEN expression was detected at 4 hours. An average increase in expression of ~2-times was observed at 8 hours and maintained over 24 hours compared to the controls.

Figure 13.3 shows PAI-1 mRNA expression in response to cytokine stimulation. At 4 hours, a ~3-fold increase in expression compared to the controls was observed. At 8 and 24 hours the levels of PAI-1 expression diminished but was still detectable after 24 hours, with ~2-times the level of expression remaining at 24 hours compared to samples stimulated with 2mg/ml of cytokine.

Expression levels of GAPDH shown in Figure 13.2 are constant throughout, and all RT-PCR controls were free of contamination as shown in Figure 13.4. The results observed in Figures 13.1 to 13.3 were again shown by densitometric analysis of band intensities (Table 6).

Table 6 Ishikawa Serum Non-starved, Experiment 1 PCR Densitometry

| PTEN PCR A | | | PTEN PCR B | | |
|-----------------------------------|------------|------------|-----------------------------------|------------|------------|
| TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD | TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD |
| 0ng/4 | 59.86 | 141 | 0ng/4 | 57.3 | 121 |
| 8 | 52.73 | 113 | 8 | 62 | 148 |
| 24 | 49.9 | 92 | 24 | 61.52 | 154 |
| 2ng/4 | 42.85 | 89 | 2ng/4 | 43.7 | 81 |
| 8 | 120.04 | 198 | 8 | 114.9 | 193 |
| 24 | 63.7 | 150 | 24 | 93.8 | 172 |
| 5ng/4 | 51.4 | 113 | 5ng/4 | 66 | 115 |
| 8 | 118 | 195 | 8 | 120.1 | 197 |
| 24 | 114.1 | 186 | 24 | 121 | 196 |
| GAPDH PCR A | | | GAPDH PCR B | | |
| TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD | TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD |
| 4 | 326.3 | 255 | 4 | 333.4 | 254 |
| 8 | 330 | 255 | 8 | 334 | 254 |
| 24 | 331.5 | 254 | 24 | 334 | 255 |
| 2ng/4 | 330.04 | 255 | 2ng/4 | 335.1 | 256 |
| 8 | 330 | 256 | 8 | 334.21 | 255 |
| 24 | 333.1 | 254 | 24 | 335 | 255 |
| 5ng/4 | 329 | 254 | 5ng/4 | 335.3 | 253 |
| 8 | 334.3 | 255 | 8 | 334 | 254 |
| 24 | 332 | 255 | 24 | 334.6 | 255 |
| PAI-1 PCR A | | | PAI-1 PCR B | | |
| TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD | TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD |
| 0ng/4 | 40.56 | 89 | 0ng/4 | 43.12 | 96 |
| 8 | 43.15 | 95 | 8 | 45.5 | 99 |
| 24 | nd | nd | 24 | nd | nd |
| 2ng/4 | 71.8 | 159 | 2ng/4 | 94.33 | 176 |
| 8 | 62.71 | 147 | 8 | 70.4 | 155 |
| 24 | 25.4 | 58 | 24 | 24.91 | 57 |
| 5ng/4 | 124 | 192 | 5ng/4 | 121.3 | 192 |
| 8 | 64.1 | 142 | 8 | 69.7 | 157 |
| 24 | 41.51 | 94 | 24 | 41.6 | 93 |

Serum non-starved Ishikawa cells, Experiment 2

| | | | | | | | | | | |
|---|---|-----|----|--|---|---|-----|--|--|--|
| | | Ong | | | | | 2ng | | | |
| S | 4 | 8 | 24 | | 4 | 8 | 24 | | | |

I 24

A

B

Fig 14.1 Expression of PTEN in non-starved Ishikawa with duplicates A and B. No TGF-pi control shown in white, 2ng/ml TGF-pi in red, 5ng/ml TGF-p1 in blue over 4, 8 & 24 hours. PCR control (no DNA) is a yellow dash.

A

B

Fig 14.2 Expression of GAPDH in non-starved Ishikawa with duplicates A and B. No TGF-pi control shown in white, 2ng/ml TGF-p1 in red, 5ng/ml TGF-pi in blue over 4, 8 & 24 hours. PCR control (no DNA) is a yellow dash.

A

B

Fig 14.3 Expression of PAI-1 in non-starved Ishikawa with duplicates A and B. No TGF-p1 control shown in white, 2ng/ml TGF-p1 in red, 5ng/ml TGF-p1 in blue over 4, 8 & 24 hours. PCR control (no DNA) is a yellow dash

Serum non-starved Ishikawa cells stimulated with TGF- β 1

This experiment was performed once, with duplicate flasks A and B. From these duplicate flask, cDNA was prepared twice on separate occasions for RT-PCR using primers for PTEN, PAI-1 and GAPDH. The two individual batches of cDNA were designated as experiments 1 and 2 respectively.

Experiment 2

Analysis of RT-PCR products shown in Figure 14.1 show the same time and dose-dependant two-fold increase in PTEN mRNA expression in samples stimulated with TGF- β 1 after 8 hours to Ishikawa non-starved Experiment 1(Figure 13.1). This confirmed that the original RT-PCR results in Experiment 1 were correct, as the fresh cDNA used in Experiment 2 gave very similar results. Expression levels of GAPDH shown in Figure 14.2 and PAI-1in Figure 14.3 are also similar to Ishikawa Experiment 1, with the results for PAI-1 showing a three-fold increase with both concentrations of TGF- β 1.

As PAI-1 expression in Ishikawa Experiment 1 was only induced 2-fold by 2ng/ml cytokine, this difference may be due to the use of a new batch of RT enzyme used in the cDNA synthesis for Ishikawa Experiment 2, causing slight variations in the cDNA. Band intensities from Figures 14.1 to 14.3 were again also shown by densitometric analysis (Table 7).

Summary of Results for Experiments 1 & 2

A modest up-regulation of PTEN mRNA was observed in cells stimulated with TGF- β 1 compared to the controls. The increase in transcripts was observed to be comparable in cells stimulated with 2ng/ml or 5ng/ml of

cytokine, which was seen to be approximately double after 8 hours compared to the unstimulated controls, and was maintained over the 24 hour period.

The results obtained for serum non-starved Ishikawa and serum non-starved HEC-1B were very similar, with a time and dose-dependant increase in PTEN mRNA observed in response to cytokine stimulation. With regard to the control samples, the Ishikawa cell line did not appear to exhibit the slight up-regulation of PTEN over 24 hours in the absence of TGF- β 1 that was seen in HEC-1B cells. This may indicate variation in the sensitivity of these two cell lines to serum components, or may be due to experimental difference, such as the source of serum used, which contains variable amounts of cytokines and other factors.

Table 7 Ishikawa Serum Non-Starved, Experiment 2 PCR Densitometry

| PTEN PCR A | | | PTEN PCR B | | |
|-----------------------------------|------------|------------|-----------------------------------|------------|------------|
| TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD | TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD |
| 0ng/4 | 61 | 146 | 0ng/4 | 62.4 | 150 |
| 8 | 59.32 | 142 | 8 | 61.73 | 150 |
| 24 | 59.4 | 145 | 24 | 61.8 | 152 |
| 2ng/4 | 64.03 | 153 | 2ng/4 | 60.11 | 155 |
| 8 | 120.6 | 201 | 8 | 125.7 | 207 |
| 24 | 127.61 | 205 | 24 | 122.96 | 206 |
| 5ng/4 | 65 | 151 | 5ng/4 | 61.3 | 150 |
| 8 | 129.01 | 209 | 8 | 126.2 | 210 |
| 24 | 126.95 | 205 | 24 | 125.58 | 207 |
| GAPDH PCR A | | | GAPDH PCR B | | |
| TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD | TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD |
| 4 | 331.2 | 254 | 4 | 327 | 253 |
| 8 | 330 | 254 | 8 | 331.5 | 254 |
| 24 | 330.4 | 253 | 24 | 332 | 254 |
| 2ng/4 | 333.02 | 256 | 2ng/4 | 330 | 255 |
| 8 | 329.7 | 255 | 8 | 330.81 | 256 |
| 24 | 330.1 | 253 | 24 | 328.97 | 255 |
| 5ng/4 | 327 | 254 | 5ng/4 | 330 | 254 |
| 8 | 331.3 | 255 | 8 | 330.6 | 253 |
| 24 | 329 | 254 | 24 | 331.72 | 254 |
| PAI-1 PCR A | | | PAI-1 PCR B | | |
| TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD | TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD |
| 0ng/4 | 54.8 | 99 | 0ng/4 | 55 | 96 |
| 8 | 57.2 | 98 | 8 | 50.3 | 97 |
| 24 | 43.01 | 51 | 24 | 45.42 | 53 |
| 2ng/4 | 196 | 239 | 2ng/4 | 199.12 | 242 |
| 8 | 112.1 | 190 | 8 | 114 | 187 |
| 24 | 40.37 | 88 | 24 | 43.6 | 92 |
| 5ng/4 | 197.5 | 238 | 5ng/4 | 201.81 | 240 |
| 8 | 113.9 | 189 | 8 | 118 | 197 |
| 24 | 64.04 | 152 | 24 | 69.11 | 154 |

3.1.2 Analysis of PTEN, Phospho-PTEN, β -Actin and PAI-1 protein levels in HEC-1B and Ishikawa cell lines stimulated with TGF- β 1

To determine whether changes in mRNA expression elicited any modification of protein expression in response to TGF- β 1 stimulation, total protein was extracted from HEC-1B and Ishikawa cells which had been treated with no cytokine or 2 and 5ng/ml TGF- β 1, again under either serum-starved or non-starved conditions (Chapter 2, Section 2.2.4.1), and levels of specific proteins (PTEN, PAI-1, phospho-PTEN [P-PTEN] and β -Actin) determined by Western blotting. Determination of levels of PTEN and P-PTEN were required to ascertain any biological significance of PTEN mRNA up-regulation by TGF- β 1.

3.1.2.1 Determination of protein concentration by the Bradford assay

To determine the concentration of protein in each sample obtained from stimulation with TGF- β 1, a BSA standard curve was first prepared according to the instructions supplied with the Bradford reagent. Quantitation of the protein was necessary to enable the equal loading of 10 μ g onto the SDS-PAGE gel from each sample, so that expression levels could be compared. Briefly, serial dilutions of BSA were made in triplicate and the absorbance recorded at 595nm using disposable cuvettes after incubation with the reagent. The readings for each dilution were averaged and the results plotted as shown in Appendix IV.

Samples obtained from the stimulation experiments were measured in duplicate as described in Chapter 2, Section 2.2.4.2 and the average values shown in Table 8 with the volumes required for 10 μ g of protein.

| Table 8 Protein Concentrations from HEC-1B & Ishikawa Stimulated with TGF- β 1 | | | | | | | | |
|--|---------------------------|---|---------------------------|---|---------------------------|---|---------------------------|---|
| Flask | HEC-1B Non-Starved | | HEC-1B Serum Starved 1 | | HEC-1B Serum-Starved 2 | | Ishikawa Non-Starved | |
| | $\mu\text{g}/\mu\text{l}$ | For 10 μg (μl) | $\mu\text{g}/\mu\text{l}$ | For 10 μg (μl) | $\mu\text{g}/\mu\text{l}$ | For 10 μg (μl) | $\mu\text{g}/\mu\text{l}$ | For 10 μg (μl) |
| 10% A | x | x | 0.75 | 13 | 0.36 | 6 | x | x |
| B | x | x | 1.64 | 6 | 0.29 | 7 | x | x |
| 0/4 A | 1.02 | 9.8 | 1 | 10 | 0.45 | 22 | 1.72 | 5.81 |
| B | 1.04 | 9.6 | 1.3 | 8.1 | 1.17 | 8.5 | 1.1 | 9.1 |
| 0/8 A | 0.94 | 10.6 | 1.5 | 6.7 | 0.24 | 42 | 1.14 | 8.77 |
| B | 0.82 | 12.2 | 1.78 | 5.1 | 0.85 | 11.76 | 1.34 | 7.46 |
| 0/24 A | 1.16 | 8.6 | 1.68 | 5.95 | 0.6 | 17 | 1.34 | 7.46 |
| B | 1.16 | 8.6 | 1.66 | 6 | 0.5 | 20 | 1.82 | 5.49 |
| 2/4 A | 1.02 | 9.8 | 1.28 | 7.8 | 0.74 | 14 | 1.2 | 8.33 |
| B | 1.02 | 9.8 | 1.54 | 6.5 | 0.7 | 14 | 1.44 | 6.94 |
| 2/8 A | 0.84 | 11.9 | 1.66 | 6 | 0.84 | 12 | 1.2 | 8.33 |
| B | 1.1 | 9.1 | 1.41 | 7.2 | 0.76 | 13 | 1.44 | 6.94 |
| 2/24 A | 0.94 | 10.6 | 1.67 | 6 | 0.6 | 17 | 1.46 | 6.85 |
| B | 0.78 | 12.8 | 1.27 | 8.3 | 0.72 | 13.8 | 1.44 | 6.94 |
| 5/4 A | 1.02 | 9.8 | 1.24 | 8.1 | 0.74 | 14 | 1.5 | 6.67 |
| B | 1.04 | 9.6 | 1.69 | 5.9 | 0.76 | 13 | 1.4 | 7.14 |
| 5/8 A | 1.1 | 9.1 | 1.72 | 5.8 | 0.7 | 14 | 1.26 | 7.93 |
| B | 1.1 | 9.1 | 1.52 | 6.5 | 0.78 | 12.8 | 1.4 | 7.14 |
| 5/24 A | 1.1 | 9.1 | 1.36 | 7.4 | 0.6 | 17 | 1.63 | 6.13 |
| B | 0.7 | 0.7 | 0.98 | 10 | 0.38 | 26 | 1.88 | 5.32 |

3.1.2.3 Detection of proteins by Western blot with enhanced chemiluminescence (ECL) and densitometry.

Western blotting and detection by ECL were performed as described in Chapter 2, Section 2.2.4 for HEC-1B and Ishikawa cell lines, after fractionation of 10µg of each protein sample by 8% SDS-PAGE against a broad-range biotinylated molecular weight marker (Appendix I.4). Antibodies were used to detect PTEN, phospho-PTEN Ser380 (P-PTEN), β -Actin and PAI-1 as described in Chapter 2.

Both PTEN and phospho-PTEN (Ser380) levels were analysed using two separate antibodies (anti-PTEN from BD Biosciences and anti-P-PTEN Ser380 from NEB). This was performed only on the HEC-1B cell line which expresses wild-type PTEN, as the Ishikawa cell line does not express PTEN protein (Figure 18.1) due to a mutation of PTEN in this cell line (Yaginuma, et al, 2000). This was to determine if TGF- β 1 was capable of modifying overall PTEN protein levels, which could indicate a potential use as an anti-tumour therapy, and also if the amount of phospho-PTEN was also changed. As phosphorylation of PTEN prevents degradation, but also inhibits catalytic activity (Vazquez, et al 2000), assessing the phosphorylation status under cytokine stimulus may aid in the elucidation of mechanisms of PTEN protein regulation by TGF- β 1.

Scanned images of the developed films are shown in Figures 15 to 18 The intensity of each specific band, indicated by an arrow, was analysed (Chapter 2, Section 2.2.4.5.1) and displayed in Tables 9 to 12.

Results for HEC-1B serum non-starved are shown in Figures 15.1 to 15.4 and Table 9.

Results for HEC-1B serum-starved Experiments 1 and 2 are shown in Figures 16.1 to 16.2, Table 10 and Figures 17.1 to 17.4 and Table 11, respectively. The results for non-serum starved Ishikawa cells are presented in Figures 18.1 to 18.3 and Table 12.

HEC-1B Serum non-starved

Figure 15.1 Anti-PTEN western blot for non-starved HEC-1B with duplicates A and B. No TGF-p1 control shown in white, 2ng/ml TGF-p1 in red, 5ng/ml TGF-p1 in blue over 4, 8 & 24 hours Arrow indicates specific band at ~55KDa

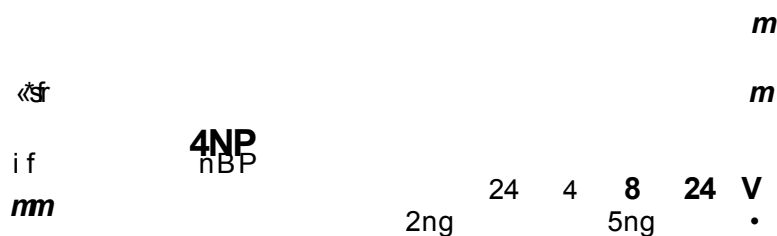


Figure 15.2 Anti-P Actin western blot for non-starved HEC-1B with duplicates A and B. No TGF-p1 control shown in white, 2ng/ml TGF-p1 in red, 5ng/ml TGF-p1 in blue over 4, 8 & 24 hours. Arrow indicates specific band at ~43KDa.

Fig 15.3 Anti-phospho-PTEN (Ser380) western blot for Non-starved HEC-1B with duplicates A and B. No TGF-p1 control shown in white, 2ng/ml TGF-pi in red, 5ng/ml TGF-pi in blue over 4, 8 & 24 hours. Arrow indicates specific band at ~55KDa.

U P

Fig 15.4 Anti-PAI-1 western blot for non-starved HEC-1B with duplicates A and B. No TGF-p1 control shown in white, 2ng/ml TGF-pi in red, 5ng/ml TGF-pi in blue over 4, 8 & 24 hours. Arrow indicates specific band at ~47KDa.

Non-specific band
^ PTEN

Fig 15.5 Anti-PTEN blot to show the source of the high molecular weight non-specific band with (+ 1°Ab) or without (-1° Ab) primary anti-PTEN antibody.
M= protein marker.

Serum non-starved HEC-1B stimulated with TGF- β 1

This experiment was performed once, with duplicate flasks A and B from which total protein was prepared for Western blotting using antibodies against for PTEN, phospho-PTEN(Ser380), PAI-1 and β -actin.

As can be seen in Figure 15.1, expression of PTEN protein in HEC-1B (band in A and B samples at ~55KDa with respect to the marker) changes little between the samples, with only a very minor decrease after 24 hours with 5ng/ml of cytokine, and does not exhibit the increase observed in mRNA (Figure 10.1). Expression of PTEN protein was not assessed in the publications describing modulation of PTEN mRNA by TGF- β 1(Lee, et al 1999 and Li and Sun, 1997), but PTEN protein was decreased in pancreatic cells after 24 hours of treatment with 10ng/ml of cytokine (Ebert, et al 2002). Levels of β -Actin in Figure 15.2 and phospho-PTEN in Figure 15.3 are also fairly constant.

It should be noted that the phospho-PTEN antibody used here is specific to Ser380. A new phospho-PTEN antibody is now available which recognises phosphorylated Ser380 and Thr382/383, and therefore could be used to detect changes at all three residues unlike the Ser380-specific antibody. The expression of PAI-1 protein (Fig 15.4) shows a similar pattern as for PAI-1 mRNA (Figure 10.3), with a time and dose-dependant increase of ~4-fold at 4 hours rising to ~5-fold at 24 hours for both concentrations of TGF- β 1. Band intensities were also shown by densitometric analysis (Table 9).

The high molecular weight band of >90KDa observed in variable amounts in all the blots was due to non-specific binding by the ABC/secondary antibody complex. This band was present even in the absence of primary anti-PTEN

antibody (Figure 15.5), and therefore was not an artefact of non-specific binding by the primary antibody.

Summary of Results

Expression of PTEN and P-PTEN did not appear to undergo any detectable change in levels in cells stimulated with TGF- β 1 compared to the controls. This would indicate that although the cytokine was capable of up-regulating PTEN mRNA, no change in protein levels were observed suggesting that TGF- β 1 does not represent a novel anti-tumour therapy in the HEC-1B cell line.

| Anti-PTEN A | | | Anti-PTEN B | | |
|-----------------------------------|-----------|------------|-----------------------------------|-----------|------------|
| TGF- β 1(ng) /Time (hrs) | Ave OD | Peak OD | TGF- β 1(ng) /Time (hrs) | Ave OD | Peak OD |
| 0ng/4 | 0.42 | 0.52 | 0ng/4 | 0.36 | 0.48 |
| 8 | 0.39 | 0.5 | 8 | 0.38 | 0.52 |
| 24 | 0.45 | 0.58 | 24 | 0.35 | 0.46 |
| 2ng/4 | 0.45 | 0.55 | 2ng/4 | 0.37 | 0.5 |
| 8 | 0.43 | 0.53 | 8 | 0.34 | 0.43 |
| 24 | 0.39 | 0.49 | 24 | 0.38 | 0.51 |
| 5ng/4 | 0.37 | 0.42 | 5ng/4 | 0.35 | 0.5 |
| 8 | 0.35 | 0.43 | 8 | 0.32 | 0.44 |
| 24 | 0.34 | 0.42 | 24 | 0.25 | 0.32 |
| Anti- β Actin A | | | Anti- β Actin B | | |
| TGF- β 1(ng) /Time (hrs) | Ave OD | Peak OD | TGF- β 1(ng) /Time (hrs) | Ave OD | Peak OD |
| 0ng/4 | 0.48 | 0.73 | 0ng/4 | 0.46 | 0.74 |
| 8 | 0.5 | 0.75 | 8 | 0.5 | 0.82 |
| 24 | 0.55 | 0.85 | 24 | 0.53 | 0.85 |
| 2ng/4 | 0.52 | 0.77 | 2ng/4 | 0.54 | 0.85 |
| 8 | 0.51 | 0.78 | 8 | 0.57 | 0.87 |
| 24 | 0.44 | 0.7 | 24 | 0.53 | 0.74 |
| 5ng/4 | 0.45 | 0.7 | 5ng/4 | 0.45 | 0.7 |
| 8 | 0.44 | 0.68 | 8 | 0.4 | 0.64 |
| 24 | 0.43 | 0.67 | 24 | 0.42 | 0.65 |

| Anti-P-PTEN A | | | Anti-P-PTEN B | | |
|-----------------------------------|------------|------------|-----------------------------------|------------|------------|
| TGF- β 1(ng) /Time (hrs) | Ave OD | Peak OD | TGF- β 1(ng) /Time (hrs) | Ave OD | Peak OD |
| 0ng/4 | 0.54 | 0.73 | 0ng/4 | 0.52 | 0.69 |
| 8 | 0.55 | 0.73 | 8 | 0.56 | 0.68 |
| 24 | 0.62 | 0.87 | 24 | 0.65 | 0.73 |
| 2ng/4 | 0.64 | 0.8 | 2ng/4 | 0.67 | 0.75 |
| 8 | 0.6 | 0.82 | 8 | 0.65 | 0.71 |
| 24 | 0.62 | 0.82 | 24 | 0.66 | 0.7 |
| 5ng/4 | 0.58 | 0.76 | 5ng/4 | 0.53 | 0.67 |
| 8 | 0.45 | 0.6 | 8 | 0.55 | 0.64 |
| 24 | 0.5 | 0.65 | 24 | 0.49 | 0.6 |
| Anti-PAI-1 A | | | Anti-PAI-1 B | | |
| TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD | TGF- β 1(ng) /Time (hrs) | Mass ng | Peak OD |
| 0ng/4 | 0.13 | 0.14 | 0ng/4 | nd | nd |
| 8 | 0.14 | 0.15 | 8 | 0.12 | 0.12 |
| 24 | 0.13 | 0.14 | 24 | 0.11 | 0.12 |
| 2ng/4 | 0.37 | 0.45 | 2ng/4 | 0.41 | 0.53 |
| 8 | 0.38 | 0.44 | 8 | 0.43 | 0.55 |
| 24 | 0.35 | 0.42 | 24 | 0.54 | 0.68 |
| 5ng/4 | 0.25 | 0.3 | 5ng/4 | 0.42 | 0.54 |
| 8 | 0.28 | 0.37 | 8 | 0.48 | 0.56 |
| 24 | 0.33 | 0.46 | 24 | 0.57 | 0.66 |

Table 9 HEC-1B Serum non-starved Western blot Densitometry

HEC-1B Serum-starved, Experiment 1

4 8 24 4 8 24
5n9 A

Fig 16.1 Anti-PTEN western blot for serum starved HEC-1B
with duplicates A and B. No TGF-pi control shown in white, 2ng/ml TGF-p1
in red, 5ng/ml TGF-p1 in blue over 4, 8 & 24 hours. Arrow indicates specific
band at ~55KDa.

Fig 16.2 Anti-β actin western blot for serum starved HEC-1B with
duplicates A and B. No TGF-p1 control shown in white, 2ng/ml TGF-pi in
red, 5ng/ml TGF-pi in blue over 4, 8 & 24 hours. Arrow indicates specific
band at ~43KDa.

| Table 10 HEC-1B Serum Starved Experiment 1 Western blot Densitometry | | | | | |
|---|-----------|------------|-----------------------------------|-----------|------------|
| Anti-PTEN A | | | Anti-PTEN B | | |
| TGF- β 1(ng) /Time (hrs) | Ave OD | Peak OD | TGF-1(ng) /Time (hrs) | Ave OD | Peak OD |
| 10% | 0.18 | 0.23 | 10% | 0.26 | 0.34 |
| 0ng/4 | 0.22 | 0.33 | 0ng/4 | 0.24 | 0.35 |
| 8 | 0.24 | 0.35 | 8 | 0.24 | 0.35 |
| 24 | 0.23 | 0.35 | 24 | 0.24 | 0.35 |
| 2ng/4 | 0.28 | 0.37 | 2ng/4 | 0.4 | 0.52 |
| 8 | 0.35 | 0.47 | 8 | 0.44 | 0.59 |
| 24 | 0.32 | 0.44 | 24 | 0.4 | 0.55 |
| 5ng/4 | 0.31 | 0.45 | 5ng/4 | 0.4 | 0.56 |
| 8 | 0.33 | 0.48 | 8 | 0.43 | 0.6 |
| 24 | 0.25 | 0.36 | 24 | 0.39 | 0.54 |
| Anti- β Actin A | | | Anti- β Actin B | | |
| TGF- β 1(ng) /Time (hrs) | Ave OD | Peak OD | TGF- β 1(ng) /Time (hrs) | Ave OD | Peak OD |
| 10% | 0.45 | 0.68 | 10% | 0.5 | 0.77 |
| 0ng/4 | 0.53 | 0.75 | 0ng/4 | 0.51 | 0.74 |
| 8 | 0.55 | 0.79 | 8 | 0.46 | 0.7 |
| 24 | 0.63 | 0.86 | 24 | 0.58 | 0.89 |
| 2ng/4 | 0.52 | 0.77 | 2ng/4 | 0.6 | 0.86 |
| 8 | 0.49 | 0.76 | 8 | 0.65 | 0.91 |
| 24 | 0.5 | 0.74 | 24 | 0.65 | 0.91 |
| 5ng/4 | 0.54 | 0.82 | 5ng/4 | 0.62 | 0.9 |
| 8 | 0.54 | 0.83 | 8 | 0.48 | 0.71 |
| 24 | 0.53 | 0.78 | 24 | 0.45 | 0.68 |

HEC-1B Serum-starved experiment 2

Fig 17.1 Anti-PTEN western blot for serum starved HEC-1B
with duplicates A and B. No TGF-p1 control shown in white, 2ng/ml TGF-p1
in red, 5ng/ml TGF-pi in blue over 4, 8 & 24 hours. Arrow indicates specific
band at ~55KDa.

Fig 17.2 Anti-p actin western blot for serum starved HEC-1B
with duplicates A and B. No TGF-p1 control shown in white, 2ng/ml TGF-pi
in red, 5ng/ml TGF-pi in blue over 4, 8 & 24 hours. Arrow indicates specific
band at ~43KDa.

Fig 17.3 Anti-phospho-PTEN (Ser 380) western blot for serum starved HEC-1B with duplicates A and B. No TGF-pi control shown in white, 2ng/ml TGF-pi in red, 5ng/ml TGF-pi in blue over 4, 8 & 24 hours. Arrow indicates specific band at ~55KDa.

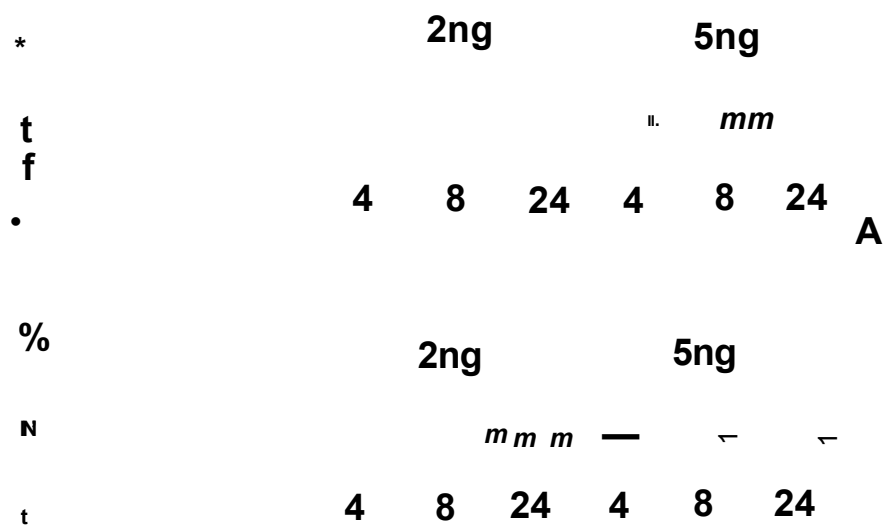


Fig 17.4 Anti-PAI-1 western blot serum starved HEC-1B with duplicates A and B. No TGF-p1 control shown in white, 2ng/ml TGF-p1 in red, 5ng/ml TGF-pi in blue over 4, 8 & 24 hours. Arrow indicates specific band at ~47KDa

Serum starved HEC-1B stimulated with TGF- β 1

This experiment was performed twice in duplicate flasks A and B from which total protein was prepared for Western blotting using antibodies against for PTEN, phospho-PTEN(Ser380), PAI-1 and β -actin. Proteins extracted in experiment 1 were scraped into cold Tris, and therefore P-PTEN could not be detected in these samples. Proteins extracted in experiment 2 were isolated using a phosphoprotein lysis buffer (Appendix I.3) and could therefore be probed for P-PTEN.

Experiments 1 & 2

The results of both serum-starved HEC-1B experiments (Figures 16.1 and 17.1) show little change in PTEN expression levels, and again β -Actin remained constant (Figures 16.2 and 17.2). A slight decrease in phospho-PTEN levels was observed in a time and dose-dependant manner (Figure 17.3), but this decrease was very small and not completely consistent between duplicate samples. A strong response was observed for PAI-1 (Figure 17.4) which was undetectable in the controls. A continuous increase from 4 to 24 hours of stimulation was seen, giving approximately double the band intensity after 24 hours, with the strongest response from the 5ng/ml samples. These observations were reflected in the densitometry results shown in Tables 10 and 11.

Experiment 1 only included blots for PTEN and β -Actin due to the protein extraction method used (scrapping into ice-cold Tris). The cell lysates initially prepared in this manner were very viscous, which gave difficulty loading onto the gels, and upon probing for phospho-PTEN were seen to have degraded

substantially. Phosphoprotein lysis buffer (protocol courtesy of Mr Mike Sharrard, University of York), was later employed to overcome this problem.

Summary of Results for experiments 1 & 2

Expression of PTEN and P-PTEN did not appear to undergo any detectable change in cells stimulated with TGF- β 1 compared to the controls. This would indicate that although the cytokine was capable of up-regulating PTEN mRNA, no change in protein levels was observed. Reduction of foetal calf serum from 10% to 1% in these experiments also appear to have very little effect on PTEN and P-PTEN expression, even though reduction of serum was observed to mildly induce expression of PTEN mRNA (Figures 11 and 12, Section 3.1.1).

Again, as PTEN and P-PTEN levels remained unchanged with cytokine stimulation, TGF- β 1 does not appear to represent an anti-tumour agent in this cell line.

**Table 11 HEC-1B Serum starved Experiment 2
Western blot Densitometry**

| Anti-PTEN A | | | Anti-PTEN B | | |
|--|-----------|------------|--|-----------|------------|
| TGF- β 1(ng) /Time (hrs) | Ave OD | Peak OD | TGF- β 1(ng) /Time (hrs) | Ave OD | Peak OD |
| 10% | 0.29 | 0.37 | 10% | 0.17 | 0.21 |
| 0ng/4 | 0.44 | 0.63 | 0ng/4 | 0.35 | 0.49 |
| 8 | 0.42 | 0.59 | 8 | 0.37 | 0.52 |
| 24 | 0.54 | 0.75 | 24 | 0.45 | 0.65 |
| 2ng/4 | 0.58 | 0.8 | 2ng/4 | 0.43 | 0.61 |
| 8 | 0.49 | 0.69 | 8 | 0.41 | 0.61 |
| 24 | 0.48 | 0.62 | 24 | 0.4 | 0.55 |
| 5ng/4 | 0.45 | 0.56 | 5ng/4 | 0.38 | 0.52 |
| 8 | 0.56 | 0.74 | 8 | 0.37 | 0.53 |
| 24 | 0.48 | 0.71 | 24 | 0.42 | 0.49 |
| Anti-β Actin A | | | Anti-β Actin B | | |
| TGF- β 1(ng) /Time (hrs) | Ave OD | Peak OD | TGF- β 1(ng) /Time (hrs) | Ave OD | Peak OD |
| 10% | 0.7 | 0.94 | 10% | 0.52 | 0.75 |
| 0ng/4 | 0.74 | 0.96 | 0ng/4 | 0.53 | 0.69 |
| 8 | 0.75 | 1.01 | 8 | 0.61 | 0.81 |
| 24 | 0.78 | 1.04 | 24 | 0.60 | 0.65 |
| 2ng/4 | 0.61 | 0.85 | 2ng/4 | 0.63 | 0.67 |
| 8 | 0.64 | 0.84 | 8 | 0.69 | 0.79 |
| 24 | 0.65 | 0.82 | 24 | 0.63 | 0.83 |
| 5ng/4 | 0.6 | 0.76 | 5ng/4 | 0.65 | 0.80 |
| 8 | 0.56 | 0.71 | 8 | 0.6 | 0.75 |
| 24 | 0.48 | 0.68 | 24 | 0.62 | 0.72 |

| Anti-P-PTEN A | | | Anti-P-PTEN B | | |
|-----------------------------------|-----------|------------|-----------------------------------|-----------|------------|
| TGF- β 1(ng) /Time (hrs) | Ave OD | Peak OD | TGF- β 1(ng) /Time (hrs) | Ave OD | Peak OD |
| 10% | 0.23 | 0.31 | 10% | 0.22 | 0.27 |
| 0ng/4 | 0.35 | 0.51 | 0ng/4 | 0.28 | 0.38 |
| 8 | 0.36 | 0.53 | 8 | 0.27 | 0.37 |
| 24 | 0.37 | 0.58 | 24 | 0.34 | 0.44 |
| 2ng/4 | 0.36 | 0.55 | 2ng/4 | 0.33 | 0.43 |
| 8 | 0.29 | 0.46 | 8 | 0.3 | 0.38 |
| 24 | 0.25 | 0.41 | 24 | 0.24 | 0.31 |
| 5ng/4 | 0.25 | 0.39 | 5ng/4 | 0.24 | 0.31 |
| 8 | 0.25 | 0.39 | 8 | 0.27 | 0.35 |
| 24 | 0.21 | 0.33 | 24 | 0.29 | 0.37 |
| Anti-PAI-1 A | | | Anti-PAI-1 B | | |
| TGF- β 1(ng) /Time (hrs) | Ave OD | Peak OD | TGF- β 1(ng) /Time (hrs) | Ave OD | Peak OD |
| 0ng/4 | nd | nd | 0ng/4 | nd | nd |
| 8 | nd | nd | 8 | nd | nd |
| 24 | nd | nd | 24 | nd | nd |
| 2ng/4 | 0.29 | 0.39 | 2ng/4 | 0.38 | 0.46 |
| 8 | 0.49 | 0.65 | 8 | 0.5 | 0.6 |
| 24 | 0.65 | 0.93 | 24 | 0.69 | 0.91 |
| 5ng/4 | 0.37 | 0.47 | 5ng/4 | 0.42 | 0.48 |
| 8 | 0.58 | 0.71 | 8 | 0.65 | 0.78 |
| 24 | 0.72 | 0.99 | 24 | 0.83 | 1.0 |

Table 11 (Continued)

HEC-1B Serum starved Experiment 2 Western blot Densitometry

Ishikawa Serum Non-Starved

Fig 18.1 Anti-PTEN Western blot for non-starved Ishikawa & HEC-1B

H=HEC-1B, I=Ishikawa, arrow indicates specific band at ~54KDa

| <i>m</i> | 2ng | | | 5ng | | |
|----------|-----|---|----|-----|---|----|
| * | 4 | 8 | 24 | 4 | 8 | 24 |

Fig 18.2 Anti-PAI-1 western blot for non-starved Ishikawa

with duplicates A and B. No TGF-pi control shown in white, 2ng/ml TGF-pi in red, 5ng/ml TGF-pi in blue over 4, 8 & 24 hours. Arrow indicates specific band at ~47KDa

| | | | | |
|---|-----|---|-----|----|
| W | 24 | 4 | 8 | 24 |
| | 2ng | | 5ng | g |

Fig 18.3 Anti-p actin western blot for non-starved Ishikawa with duplicates A and B. No TGF-(31 control shown in white, 2ng/ml TGF-pi in red, 5ng/ml TGF-p1 in blue over 4, 8 & 24 hours. Arrow indicates specific band at ~43KDa

Serum non-starved Ishikawa stimulated with TGF- β 1

This experiment was performed once in duplicate flasks A and B from which total protein was prepared for Western blotting using antibodies against PTEN, phospho-PTEN(Ser380), PAI-1 and β -actin.

The absence of PTEN protein in the Ishikawa cell line was confirmed by anti-PTEN Western blot using 10 μ g of protein from HEC-1B as a positive control and an equivalent amount of protein from Ishikawa cells. The blot shown in Figure 18.1 demonstrates the lack of detectable PTEN protein in the Ishikawa cell lysate, whilst the 54KDa band (black arrow) is visible in the HEC-1B cell lysate, and confirms previous reports (Guzeloglu-Kayishli, et al 2003).

The results of the Ishikawa serum non-starved experiment showed a more moderate response for PAI-1 (Figure 18.2), but with an inverse pattern compared to HEC-1B serum non-starved (Figure 15.4). For the Ishikawa cell line, PAI-1 induction by TGF- β 1 occurred rapidly after 4 hours, then showed a steady decline up to 24 hours, where the signal is no longer detectable. This pattern of expression was also observed for mRNA expression levels shown in Figures 13 and 14, Section 3.1.1. Levels of β -actin protein remained constant throughout (Figure 18.3). These observations were verified by the densitometric analysis shown in Table 12.

Summary of Results

Expression of PTEN protein was absent in this cell line, in agreement with published material. A strong response to TGF- β 1 was observed as a robust up-regulation of PAI-1 protein, reflecting the mRNA response previously

observed in Section 3.1.1, indicating a functional signalling pathway through the TGF- β 1 receptor. The results for PAI-1 mRNA and protein up-regulation in both HEC-1B and Ishikawa cell lines indicates that the cytokine is capable of affecting PTEN mRNA expression regardless of whether PTEN is wild-type (HEC-1B) or mutated (Ishikawa).

| Table 12 Ishikawa Non-serum starved Western blot Densitometry | | | | | |
|--|-----------|------------|--------------------------|-----------|------------|
| Anti-PAI-1 A | | | Anti-PAI-1 B | | |
| TGF-b1(ng)/Time (hrs) | Ave OD | Peak OD | TGF-b1(ng)/Time (hrs) | Ave OD | Peak OD |
| 0ng/4 | 0.19 | 0.23 | 0ng/4 | 0.23 | 0.26 |
| 8 | 0.16 | 0.2 | 8 | 0.18 | 0.22 |
| 24 | nd | nd | 24 | nd | nd |
| 2ng/4 | 0.31 | 0.47 | 2ng/4 | 0.39 | 0.58 |
| 8 | 0.24 | 0.36 | 8 | 0.30 | 0.49 |
| 24 | nd | nd | 24 | nd | nd |
| 5ng/4 | 0.3 | 0.41 | 5ng/4 | 0.4 | 0.55 |
| 8 | 0.24 | 0.38 | 8 | 0.33 | 0.4 |
| 24 | nd | nd | 24 | nd | nd |
| Anti- β -Actin A | | | Anti- β -Actin B | | |
| TGF-b1(ng)/Time (hrs) | Ave OD | Peak OD | TGF-b1(ng)/Time (hrs) | Ave OD | Peak OD |
| 0ng/4 | 0.59 | 0.74 | 0ng/4 | 0.72 | 1.01 |
| 8 | 0.63 | 0.88 | 8 | 0.7 | 0.98 |
| 24 | 0.61 | 0.83 | 24 | 0.67 | 0.91 |
| 2ng/4 | 0.63 | 0.79 | 2ng/4 | 0.71 | 0.99 |
| 8 | 0.65 | 0.8 | 8 | 0.7 | 0.95 |
| 24 | 0.68 | 0.84 | 24 | 0.69 | 0.9 |
| 5ng/4 | 0.58 | 0.79 | 5ng/4 | 0.73 | 0.97 |
| 8 | 0.6 | 0.81 | 8 | 0.68 | 0.94 |
| 24 | 0.59 | 0.7 | 24 | 0.71 | 0.96 |

3.1.3 HEC-1B cell proliferation assay

From the results obtained in Section 3.1.1, further investigations were carried out to ascertain the relationship between changes in PTEN mRNA levels observed in the HEC-1B cell line and the growth of the cells under TGF- β 1 stimulation. To determine the effect of TGF- β 1 on the ability of HEC-1B cells to proliferate a simple, non-radioactive commercial cell proliferation assay was performed. This assay measures cellular reduction of the CellTitre reagent by the mitochondria of living cells to a coloured compound which can be measured using a standard plate reader. This method is an alternative to established techniques for measuring DNA synthesis such as ^3H -thymidine and 5-bromo-2-deoxyuracil (BrdU) incorporation.

HEC-1B cells were cultured on the wells of 24-well tissue culture plates in complete medium prior to serum starvation for 24 hours in reduced serum medium. The cells were stimulated with 1 to 10ng/ml of TGF- β 1 for 4, 8 and 24 hours prior to addition of the assay reagent as described in Chapter 2, Section 2.2.5. Controls included no TGF- β 1 and no cells (media only) and all readings were taken in quadruple from this experiment, which was performed once. Mean values for the quadruple readings were subtracted from the 'no cells' control and shown in bold, (Table 13); the results were also shown graphically (Graph 4).

The cell proliferation assay for HEC-1B cells stimulated with 1-10ng/ml TGF- β 1 indicate no significant change in proliferation levels compared to the controls. Using this assay, TGF- β 1 appeared to have no positive or negative effects on proliferation levels of HEC-1B over the time course and concentration of cytokine used.

| Table 13 HEC-1B Cell Proliferation Assay Results | | | | | | | | |
|--|--------------------|------------------------------|------|------|------|------|------|------|
| Time (hours) | Ave no cells (n=4) | TGF-β1 concentration (ng/ml) | | | | | | |
| | | 0 | 1 | 2 | 4 | 5 | 7 | 10 |
| 4 | 0.134 | 1.15 | 1.07 | 1.16 | 1.00 | 0.92 | 1.05 | 0.94 |
| | | 1.11 | 1.12 | 0.99 | 0.97 | 0.94 | 1.07 | 0.99 |
| | | 1.10 | 1.15 | 0.92 | 0.92 | 1.10 | 1.09 | 0.99 |
| | | 0.96 | 1.17 | 1.01 | 1.01 | 0.92 | 0.96 | 0.94 |
| Average A490 nm (n=4) | | 1.08 | 1.1 | 0.83 | 0.98 | 0.97 | 1.04 | 0.97 |
| Average - no cells A490 nm | | 0.95 | 0.97 | 0.70 | 0.84 | 0.84 | 0.91 | 0.83 |
| 8 | 0.1437 | 1.18 | 1.04 | 1.03 | 1.26 | 1.07 | 1.06 | 1.42 |
| | | 1.25 | 1.11 | 1.12 | 1.05 | 1.14 | 1.33 | 1.04 |
| | | 1.28 | 1.28 | 1.16 | 1.02 | 1.19 | 1.05 | 1.10 |
| | | 1.27 | 1.27 | 1.23 | 1.06 | 1.25 | 1.28 | 1.0 |
| Average A490 nm (n=4) | | 1.26 | 1.17 | 1.13 | 1.1 | 1.16 | 1.18 | 1.16 |
| Average - no cells A490 nm | | 1.12 | 1.03 | 0.99 | 0.95 | 1.02 | 1.04 | 1.02 |
| 24 | 0.1387 | 1.57 | 1.38 | 1.39 | 1.28 | 1.41 | 1.40 | 1.53 |
| | | 1.75 | 1.62 | 1.18 | 1.52 | 1.65 | 1.37 | 1.68 |
| | | 1.49 | 1.81 | 1.68 | 1.49 | 1.32 | 1.25 | 1.31 |
| | | 1.67 | 1.53 | 1.46 | 1.47 | 1.61 | 1.65 | 1.48 |
| Average A490 nm (n=4) | | 1.62 | 1.58 | 1.43 | 1.44 | 1.50 | 1.42 | 1.50 |
| Average - no cells A490 nm | | 1.48 | 1.44 | 1.29 | 1.30 | 1.36 | 1.28 | 1.36 |

3.1.4 Assessment of the effect of cell density on PTEN expression in HEC-1B cells.

To determine whether the minor increase in PTEN mRNA expression observed in the HEC-1B cell line in the absence of cytokine stimulation (Figures 10, 11 and 12) could be due to increasing cell density and contact inhibition, rather than TGF- β 1, cells were cultured at low or high initial densities for several days. The effect of increasing cell density on PTEN expression was performed as described in Chapter 2, Section 2.2.6. Briefly, HEC-1B cells seeded at 4×10^5 or 9×10^5 cells per flask were cultured for 4 days in CM, or for 4 days with a media change on day 3. The media change was included to show the effects of replenishment of fresh CM, containing unknown quantities of cytokines, on PTEN expression. RNA was extracted for RT-PCR as previously detailed in Chapter 2, Section 2.2.3. Results are shown in Appendix VIII and Figures 19 to 21.

The expression of PTEN mRNA shown by RT-PCR in Figure 19 demonstrates a steady increase of PCR product over days 1 to 4, with the highest expression levels observed for the samples which received a media change on day 3 (MC). The increase in PTEN expression in day 3 MC samples may be due to renewal of stimulating factors, such as TGF- β 1 in the fresh medium. No difference was discernable between cells seeded at 4×10^5 or 9×10^5 cells per flask, therefore initial density had no effect on PTEN expression levels, which increased in a linear manner for both densities, with comparable band intensities. Figure 20 shows that no changes in expression of the internal control GAPDH occurred over the course of the experiment, and Figure 21 indicates that no DNA was present as a contaminant in the cDNA.

3.1.5 Morphological changes in HEC-1B and Ishikawa cell lines in response to TGF- β 1

As cell density alone did not appear to affect PTEN expression levels (Section 3.1.4), the effects of TGF- β 1 on the morphology of both Ishikawa and HEC-1B cell lines was evaluated. Although the cytokine did not modify PTEN protein levels in the experiments described previously, decreased proliferation and increased cell volume has been described in HEC-1B stimulated with TGF- β 1 (Boyd & Kaufman, 1990). This may involve changes in phospho-PTEN levels which could possibly be detected with the new commercial anti-phospho-PTEN (Ser380/Thr383/384) antibody. A decrease in P-PTEN levels may indicate increased PTEN protein activity and degradation and a decrease in Akt-mediated proliferation.

HEC-1B and Ishikawa cell lines were cultured in medium containing 10% foetal calf serum, then cultured for a further 48 hours in medium containing 1% serum to reduce any effects of TGF- β 1 in the medium. Cells were then cultured in reduced-serum medium in the absence or presence of TGF- β 1 at a concentration of 10ng/ml for 48 and 72 hours as described in Chapter 2, Section 2.2.7. This experiment was performed once, with triplicate wells. The live cells were photographed under phase contrast using Polaroid film (Figures 22 to 25). The cells were trypsinised and counted using a haemocytometer and the average figures from the triplicate wells are shown in Graphs 5 and 6.

HEC-1B Morphological Changes

Fig 22 Morphological changes in HEC-1B under reduced-serum conditions with no TGF- β 1 at A: 48 hours and B: 72 hours Live cells visualised after 48 & 72 hours using phase contrast microscopy. Magnification x20

Fig 23 Morphological changes in HEC-1B with 10ng/ml TGF- β 1 at A: 48 hours and B: 72 hours Live cells visualised after 48 & 72 hours using phase contrast microscopy. Magnification x200

Table 14 HEC-1B cell count x 10⁴

| | 48 hours | 72 hours |
|--|-----------------|-----------------|
| | 13.5 | 28.5 |
| No TGF-β1 | 12 | 36.5 |
| | 11.5 | 26 |
| Average | 12.3 | 30 |
| | 8 | 17 |
| 10ng/ml TGF-β1 | 8.5 | 15 |
| | 7 | 14.5 |
| Average | 7.8 | 15.5 |

[illegible]

WHX s||ao ON

By comparing Figure 22 and 23, it can be seen that treatment of HEC-1B cells with TGF- β 1 had the effect of increasing cell volume. Untreated cells were small and rounded, with a characteristic “cobblestone” appearance. After 48 hours of stimulation the cells were significantly larger, and after 72 hours had assumed a flattened profile. After performing cell counts of the stimulated and unstimulated HEC-1B cells (Table 14), the results were expressed in Graph 5.

After both 48 and 72 hours there was almost twice the number of untreated cells to treated cells. Untreated control cell numbers increased approximately 2.5 times between the 48 and 72 hour time points, whereas the TGF- β 1 treated cells increased by 2 times. In this experiment using HEC-1B cells, TGF- β 1 had the overall effect of increasing cell volume and decreasing proliferation. This effect of TGF- β 1 on HEC-1B morphology and growth has previously been reported by Boyd & Kaufman, 1990.

Ishikawa morphological changes

Fig 24 Morphological changes in Ishikawa under reduced-serum conditions with no TGF-p1 at A: 48 hours and B: 72 hours Live cells visualised after 48 & 72 hours using phase contrast microscopy. Magnification x200

Fig 25 Morphological changes in Ishikawa with 10ng/ml TGF-pi at A: 48 hours and B: 72 hours Live cells visualised after 48 & 72 hours using phase contrast microscopy. Magnification x200

Table 15 Ishikawa cell count x 104

| | 48 hours | 72 hours |
|----------------|----------|----------|
| | 14 | 16.5 |
| No TGF-pi | 12 | 18 |
| | 13.5 | 19.5 |
| Average | 13.2 | 18 |
| | 12 | 17.5 |
| 10ng/ml TGF-pi | 10.5 | 18 |
| | 10.5 | 13.5 |
| Average | 11 | 16.3 |

By comparing Figure 24 and 25, it can be seen that treatment with TGF- β 1 had little effect on Ishikawa cell volume, but after 48 hours of stimulation small patches of cells appeared to have detached from the flask, leaving areas free of cell growth.

After performing cell counts of the stimulated and unstimulated Ishikawa cells (Table 15), the results were expressed in Graph 6. After both 48 and 72 hours there was only a very slight increase in the number of untreated cells to treated cells. Additionally, little change was observed between the 48 and 72 hour time points for both treated and untreated cells. This suggests that TGF- β 1 has significantly less effect on the morphology and proliferation of Ishikawa cells compared to HEC-1B cells under the conditions used. ISK cells have previously been reported to show inhibition of proliferation in response to TGF- β 1 (Anzai, et al 1992).

3.2 CLONING OF FULL LENGTH HUMAN PTEN INTO CMV-pEGFP-N1 AND SUBCELLULAR LOCALISATION OF PTEN PROTEIN

The localisation of PTEN has been described as mainly cytoplasmic but has also been reported as nuclear in some cells, and its localisation has not previously been described in HEC-1B or Ishikawa endometrial cell lines. Changes in the localisation of PTEN in the cell are a possible mechanism of regulating its function.

To enable the rapid study of PTEN localisation in various cell lines, full length human PTEN was cloned into a GFP plasmid vector to create a C-terminal fusion protein which can be detected by fluorescent microscopy using a standard FITC filter. The production of GFP-PTEN permitted the transfection of cultured cells, regardless of the state of endogenous PTEN, and the subsequent ability to add stimulating compounds to these transiently transfected cells. The fusion protein could be rapidly visualised without the need for detection using antibodies, which may be difficult to detect if cellular levels of PTEN are very low.

The overall cloning strategy is outlined in Chapter 2, Section 2.3.1. Briefly, full-length PTEN amplified by PCR and the CMV-PEGFP-N1 vector were subjected to double-digestion with two restriction enzymes, *EcoRI* and *Sall*, to create a single cohesive end from each enzyme on both DNA sources. Subsequent ligation of the cut fragments to form a EGFP-PTEN chimera can only occur in one direction, thus ensuring that the PTEN fragment is inserted into the vector and later transcribed and translated in the correct orientation (5'→3').

The detection of other cellular compartments, proteins or organelles could also be accomplished in transfected cells using a primary antibody bound by a secondary antibody linked to a fluorescent moiety that is not green, eg red, cyan or magenta. This would enable the study of the localisation of GFP-PTEN to such cellular compartments by overlapping the two differently coloured images and noting areas of co-localisation, indicated by a colour change (eg an overlap of green and red is seen as yellow).

3.2.1 Extraction of RNA from whole human blood

Total RNA was extracted from the whole blood of four members of staff using TRI Reagent. The yield and purity of each sample was assessed by reading the absorbance at 260 and 280nm and the results shown in Table 16. Peripheral blood was chosen because PTEN mutations in lymphocytes are very rare even in haematological malignancies, therefore cells from healthy individuals was most likely to be wild-type for PTEN.

| Table 16 Staff total RNA yield and purity | | | | |
|---|--------|--------|-------|-------|
| Staff Number | A260nm | A280nm | ratio | µg/µl |
| 1 | 0.065 | 0.033 | 1.74 | 0.52 |
| 2 | 0.101 | 0.059 | 1.84 | 0.81 |
| 3 | 0.085 | 0.046 | 1.816 | 0.68 |
| 4 | 0.082 | 0.044 | 1.832 | 0.66 |

3.2.2 RT-PCR of full-length human PTEN for cloning

3.2.2.1 Analytical scale preparation of cDNA and digestion with restriction endonucleases *Mse* I and *Hha* I

cDNA was synthesised as described in Chapter 2, Section 2.3.3 from each of the four staff members and amplified using PTEN primers 1 and 4 to yield a

450bp fragment, shown in Figure 26. This fragment can be used in an analytical digest with *Mse* I and *Hha* I to distinguish between PTEN derived from pure RNA, (contains an *Mse* I site only), or from genomic contamination, (contains a *Hha* I site only). The PCR products were therefore digested with *Mse* I and *Hha* I (Figure 27). From this gel it can be clearly observed that all four PCR products were only digested with *Mse* I, and were therefore derived from PTEN cDNA and not genomic Ψ PTEN. Staff cDNA number 4 was selected for cloning as it gave a good yield of PCR product and the RNA from which it was derived had a high purity ratio (260/280nm).

3.2.2.2 PCR amplification of PTEN with high fidelity *Pfx* polymerase

cDNA was used in the amplification of full-length PTEN as described in Section 2.3.4 using the Pfx3lx PCR programme (Appendix V), and the reactions analysed by agarose gel electrophoresis (see Figure 28). To improve the yield of PCR product, which was poor, a modified version of the programme (Pfx3lx2) was then used which included a 20 minute final extension (Appendix V). The products were again analysed on an agarose gel as shown in Figure 29, and a significant improvement in yield was observed. Several PCR reactions were performed, pooled, and separated on an agarose gel to provide adequate DNA for cloning. The 1.2Kb band was carefully excised from the gel and purified as described in Chapter 2, Section 2.3.5.

Analytical RT-PCRs & Analytical Digests of Staff PTEN cDNA Samples

M -RT-RNA 1 4 M

Figure 26
PCR of staff cDNAs (1 - 4) with PTEN primers 1&4 to amplify a 450bp fragment. RT-PCR controls (-RT & -RNA), are shown with the four staff samples. M= 100bp ladder.

M 4 3 2 1 4 3 2 1

Figure 27
Aliquots of staff PCR samples 1-4 digested with *Mse* I (red) and *Hha* I (blue). M = 100bp ladder. The products were cleaved by *Mse*I to yield two distinct bands.

PCR Amplification of Full-Length Human PTEN cDNA

M - Pfx3lx

Figure 28

Electrophoresis of Staff number 4 full-length PTEN
PCR product (1.2Kb), using Pfx3lx PCR cycle. PCR
Control shown as a dash, M = 1Kb ladder.

M - Pfx3lx2

Figure 29

Electrophoresis of Staff number 4 full-length
PTEN PCR product (1.2Kb), using Pfx3lx2 cycle.
PCR Control shown as a dash, M = 1Kb ladder.

3.2.3 Digestion and purification of the PTEN fragment for ligation

To create the restriction fragment sites for ligation into the vector, the purified PTEN fragment was digested with *EcoRI* and *SaI* as described in Chapter 2, Section 2.3.5 and cleaned up as described in the same section. To assess yield a 5µl aliquot was separated on an agarose gel alongside 5µl of double digested and purified pEGFP as shown in Figure 31. From this gel a good yield of both plasmid and insert was observed, with no extraneous bands in either sample (shown in Section 3.2.5).

3.2.5 Preparation of CMV-pEGFP-N1 plasmid for cloning

The CMV-pEGFP family of plasmids are widely used for the fluorescent tagging of proteins to be expressed in mammalian cells, and are available for either N or C-terminal fusions. They also include a range of colours, such as ECFP (cyan) and EYFP (yellow) which are point mutants of the standard EGFP (green) fluorescent protein. The vectors are suitable for mammalian expression due to the presence of the strong cytomegalovirus (CMV) promoter. The EGFP-N1 version of the plasmid was chosen for cloning as this was readily available and would attach the GFP moiety to the C-terminus of the mature PTEN protein. As it has been postulated that degradation of PTEN is controlled via the C-terminus, the generation of this clone may yield some insight into the cellular effects of interfering with this process.

3.2.5.1 Transformation of *E.coli* with CMV-pEGFP-N1 and plasmid DNA isolation by Alkaline Lysis

Chemically competent JA221 cells were prepared and transformed with CMV-pEGFP-N1, as described in Chapter 2, Section 2.3.6. Numerous colonies were observed on the selective plate. Plasmid DNA extracted from a single colony by the method described in Chapter 2, Section 2.3.7 was analysed on a 1% agarose gel. 1µl of plasmid was run with a 1Kb marker as illustrated in Figure 30. The preparation was seen to consist of two bands; the upper of these being the open circular form of the plasmid and the lower being the supercoiled form. 5µl of plasmid (~10µg) was used in double restriction digest and purification as described in Chapter 2, Section 2.3.7.

3.2.5.2 Comparison of yield of PTEN and linearised pEGFP after digestion.

To give an estimation of yield of the double digested PTEN and pEGFP after purification, 5µl of each sample was separated on an agarose gel, as shown in Figure 31. The concentration of the PTEN fragment was estimated to be approximately 2.5 times that of the pEGFP fragment. The relative amount of DNA in each sample was used to determine the amount required to perform three ligations with different ratios of vector to insert DNA.

3.2.6 Ligation

Ligations were performed at 1:1, 1:3, and 1:5 ratios of vector : insert (Table 17) based on the PTEN fragment having 2.5 times the concentration of DNA as the vector, and 10 μ l analysed on an agarose gel (Figure 32). A control ligation was performed using a 100bp marker (Chapter 2, Section 2.3.8), and is shown as an inverted image in Figure 33.

| Table 17 Ligation Reactions for pEGFP & PTEN | | |
|---|------------------|--------------------------------|
| Ligation Number | Ratio V:I | V:I (μl) |
| 1 | 1:1 | 6.5:2.5 |
| 2 | 1:3 | 6.5:7.5 |
| 3 | 1:5 | 6.5:12.5 |

Table 17 Ligation ratios and volumes of vector (v) and insert (I).

PTEN & pEGFP

M pEGFP

Fig 30

Agarose gel
electrophoresis of 1µl
pEGFP. M= 1Kb
marker.

M 1 2

Fig 31

Comparison of yield of
double digested
products 5µl each of 1:
PTEN, and 2: pEGFP.
M= 100bp marker.

Ligation & Ligation Control

M 1 2 3

Fig 32

Agarose gel
electrophoresis of
ligations 1, 2 & 3 M=
1Kb ladder.

M 1 2 M

Fig 33

Control ligations of
100bp ladder at 1: 4°C
and 2: 24°C shown as
an inverted image. M=
100bp ladder

From Figure 33 it was observed that the ligation of the 100bp ladder was most successful at 4°C, as this gave the highest yield of ligated product. The lower temperature was therefore used in ligations involving PTEN and pEGFP. Figure 32 shows the three ligation reactions and as expected all contain multiple bands, indicating that the ligation was successful insofar that the DNA fragments had been randomly joined.

3.2.7 Introduction of Recombinant Plasmid DNA into *E.coli* cells

3.2.7.1 Plating out and analysis of the transformants

The three ligation reactions were used to transform chemically competent DH5α cells which were plated onto selective L-agar as described in Chapter 2, Section 2.3.9. Chemically competent cells are available commercially and avoid the need for preparing competent cell stocks. Following overnight incubation the number of colonies on each control or ligation plate was noted and shown in Table 18. From the results tabulated it was observed that plates 1 and 5 gave the highest colony numbers. These plates correspond to the ligation ratio of 1:1.

3.2.7.2 Confirmation of the presence of full length PTEN in the recombinant (pRC-2) plasmids

To confirm that the cloning had been successful, two colonies were picked from each ligation plate (with the exception of plate 5 which contained too many colonies) into selective LB for overnight growth. Plasmids were labelled A and B for each plate. The following day plasmids were extracted as previously described in Chapter 2, Section 2.3.7. Each plasmid was digested and aliquots of cut and uncut plasmid analysed on a 1% agarose gel. Gels

with uncut plasmid (Figure 34), and double digested plasmid (Figure 35) are shown overleaf.

Table 18 Results of Transformation

| Plate number | Ligation | pi cells spread | Colonies Formed |
|--------------|----------|-----------------|-----------------|
| 1 | 1 | 20 | 71 |
| 2 | 2 | 20 | 49 |
| 3 | 3 | 20 | 43 |
| 4 | control | 20 | 130 |
| 5 | 1 | 50 | 370 |
| 6 | 2 | 50 | 170 |
| 7 | 3 | 50 | 108 |
| 8 | control | 20 | 122 |

Confirmation of the presence of Recombinant Plasmids

Containing Full-Length PTEN

Fig 34

Electrophoresis of uncut plasmids derived from clones 1A to 7B. c= uncut pEGFP, M= 1Kb marker.

Fig 35

Electrophoresis of EcoRI / Sal I double digested plasmids derived from clones 1A to 7B. c= uncut pEGFP, D= linear pEGFP, M= 1Kb marker.

All the cultures gave a high yield of plasmid DNA of approximately 1-1.5Kb higher in size when compared to the pEGFP control and 1Kb marker. Upon digestion, all plasmids yielded two bands of approximately 4.7Kb and 1.2Kb. These bands corresponded to linear pEGFP and PTEN, respectively.

3.2.7.3 Preparation of pRC-2 (Clone 6A) plasmid DNA for Sequencing

From Figure 35 it was observed that clone number 6A had the cleanest digest, as the presence of an extra band at approximately 2.5Kb was not detected. In addition, Figure 34 shows that this clone had a high proportion of supercoiled DNA (lower band) to the open circular form upper band), indicating that little damage to the plasmid DNA had occurred during preparation. To confirm the PTEN insert sequence, and to verify that the clone was in-frame with respect to the EGFP tag, the plasmid was propagated and sent for automated sequencing (Chapter 2, Section 2.3.10). The sequencing data is shown in Figure 36 and includes a single strand read with standard EGFP and CMV primers. The read confirmed that pRC-2 contained wild-type PTEN.

Date sent: Fri, 28 Jun 2002 11:47:32 +0100
Subject: LARK TECHNOLOGIES
From: Stephen Foster <stephen@larkuk.co.uk>
To: Eric Blair <g.e.blair@leeds.ac.uk>

EGFP primer

GNGGGCACCCCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATGGTGGCGACCGGTGGATCCCGGGCCCCG
CGGTACCGTC
GACTGGACTTTTGTAATTTGTGTATGCTGATCTTCATCAAAGGTTCACTCTCTGGATCAGAGTCAGTGG
TGTCAGAATA
TCTATAATGATCAGGTTCACTGTCACTAACATCTGGTGTACAGAAGTTGAACTGCTAGCCTCTGGATT
GACGGCTCCT
CTACTGTTTTGTGAAGTACAGCTTCACCTTAAATTTGGAGAAAAGTATCGGTTGGCTTTGTCTTTATT
TGCTTTGTCA
AGATCATTTTTTTGTTAAAGTAAGTACTAGATATTCCTTGTCATTATCTGCACGCTCTATACTGCAAATGC
TATCGATTTT
TTGATCACATAGACTTCCATTTTCTACTTTTTCTGAGGTTTCTCTGGTCTGGTATGAAGAATGTATTT
ACCCAAAAGT
GAAACATTTTGTCTTTTTTAGCATCTTGTTCTGTTTGTGGAAGAACTCTACTTTGATATCACACACAC
AGGTAACGGC
TGAGGGAACTCAAAGTACATGAAGTTGTCTTCCCGTCGTGTGGGTCCTGAATTGGAGGAATATATCTTCA
CCTTTAGCTG
GCAGACCACAACTGAGGATTGCAAGTTCCGCCACTGAACATTGGAATAGTTTCAAACATCATCTTGTGA
AACANAGTG
CCCACTGGTCTATAATCCAGATGATTCTTTAACAGGTAGCCTATAATAATACATAGCCGCCTCTGACT
GGGAATAGTT
ACTCCCTTTTTGGNCTCTGGCCCTTACTTCCCATANAAATCTANGGCCCTNTTGGCCCTTTAAANTTN
NCCCCGANGT
AATAATTTGCCATTTCTNTTNNCCAGTTNGTCCCTTTCCAGTTTTCNNGGNAATGGCNGCATGATNG
GCCTCNCCT
NTACCCNNGGGCAAAANCTNCCAAANGGGTTGAAAAANNNCNACCGGGGGGGGNNANGNCCCCAAAGNAT
TT

Date sent: Fri, 28 Jun 2002 11:47:32 +0100
Subject: LARK TECHNOLOGIES
From: Stephen Foster <stephen@larkuk.co.uk>
To: Eric Blair <g.e.blair@leeds.ac.uk>

CMV primer

GNNGGGCCGCNCTATATAAGCAGNAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAA
ATTAATACGA
CTCACTATAGGGAGACCCAAGCTTCGAATTCAGCATGACAGCCATCATCAAAGAGATCGTTAGCAGAAAC
AAAAGGAGAT
ATCAAGAGGATGGATTCGACTTAGACTTGACCTATATTATCCAAACATTATTGCTATGGGATTTCTGTC
AGAAAGACTT
GAAGGCGTATACAGGAACAATATTGATGATGTAGTAAGGTTTTTGGATTCAAAGCATAAAACCATTACA
AGATATACAA
TCTTTGTGCTGAAAGACATTATGACACCGCCAAATTTAATTGCAGAGTTGCACAATATCCTTTTGAAGAC
CATAACCCAC
CACAGCTAGAAGTTATCAAACCTTTTGTGAAGATCTTGACCAATGGCTAAGTGAAGATGACAATCATGT
TGCAGCAATT
CACTGTAAAGCTGGAAAGGGACGAAGTGGTGAATGATATGTGCATATTTATTACATCGGGGGCAAATTTT
TAAAGGCACA
AGAGGCCCTAGATTTCTATGGGGAAGTAAGGACCAGAGACAAAAAGGGAGTAAGTATTCCAGTCAGAGG
CGCTATGTGT
ATTATTATAGCTACCTGTTAAAGAATCATCTGGATTATAGACCAGTGGCACTGTTGTTTCACAAGATGAT
GTTTGAACT
ATTCCAATGTTTCAGTGGGCGGAAGTTGCAATCCTNAGTTTGTGGTCTGCCANCTAAAGGTGAANATTTAT
TCCTCCANTT
CAGGACCCACACGACGGGNAGACAAGTTCATGTNCTTTGANTTCCCTCAACCGTTACCTGGGTGTGGGGA
ATTNAAAANA
AANTTNTCCNCAACAAAANAGAGCTAAAAAGGNCAAATGTTNCCTTTNNGGNAANAATTCTTNNCCNG
ACCGGGGAAC
CTNAAAAANTNAAANGGGGTTTTGGGNCCAAANCTAGCCTTGGCNATNAANCNCAANANNAGNAA
TTT

Figure 36

pRC-2 Clone 6A Sequence data acquired using CMV & EGFP primers.

Although it has been widely reported that PTEN is mainly a cytoplasmic protein, evidence exists that it can localise to the plasma membrane and the nucleus in a manner which may be cell-line specific (Ginn-Pease & Eng, 2003). The observation of PTEN in the nucleus suggests that it may interact with an as yet unidentified protein to modulate transcription, but as yet there is no direct evidence to support this. In this section, the localisation and distribution of PTEN was studied in endometrial cell lines by transfection with pRC-2. The monkey cell line COS-7 was also used in initial transfections as this cell line has a very flat morphology which is easy to visualise using fluorescent microscopy. A commercial lipid-based transfection reagent was chosen to deliver pRC-2 into COS-7, HEC-1B and Ishikawa cell lines. These cationic lipid reagents bind to DNA and permit transfer across the cell membrane, and are a less time-consuming alternative methods, such as calcium phosphate.

3.2.8.1 Preparation of Ultrapure Supercoiled Plasmid DNA

Preparation of high quality, supercoiled pRC-2 plasmid DNA was performed using a Sigma miniprep kit according to the manufacturer's guidelines (Chapter 2, section 2.3.11). To assess the quality of the plasmid, a 1 μ l aliquot was analysed by agarose gel electrophoresis, as shown in Figure 37. Samples of plasmid (5 μ l) were diluted 1:200 in sterile water to measure absorbance at 260nm. A stock solution of 1pg/ μ l was prepared and small aliquots frozen.

M 1
Figure 37
Electrophoresis
of 1: pRC-2, 1 μ l.
M = 1Kb ladder

Initial transfections were performed with 0.6-1.0 μ g of pRC-2 or pEGFP and 2 μ l of Lipofectamine (LF) or Lipofectamine 2000 (LF2K), as described in Chapter 2 Section 2.3.11. Transfection into both the COS-7 and HEC-1B cell lines was highly inefficient in all conditions, with less than 10% transfectants visible on each coverslip. Many of the cells were observed to have detached from the coverslips during the fixing process of submerging in cold methanol, indicating poor adherence to the glass surface. Additionally, the presence of the fusion protein did not appear to be toxic as the nuclei of transfected cells were visibly normal and several could be seen in a state of mitosis. This experiment was performed several times in triplicate and on several separate occasions. Images were captured from wells transfected with 0.8 μ g of plasmid, (see Figures 38 and 39), and are representative of the distribution observed in all transfection conditions in each individual experiment.

Transfection of COS-7 Cell Line with pRC-2

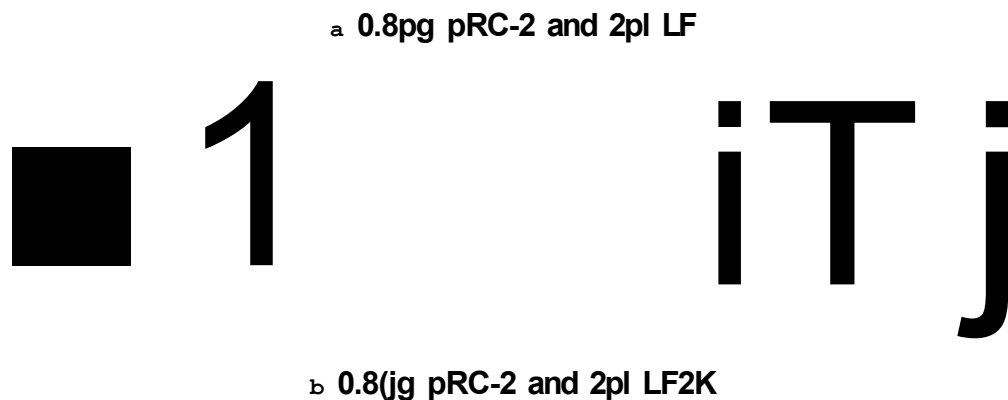


Figure 38

Transfection of COS-7 cell line with a 0.8pg pRC-2, and 2pl LF and b 0.8pg pRC-2 and 2pl LF2K. Filters used were 1 DAPI for the nucleus and 2 FITC for EGFP. Composite images are shown in 3. Magnification x400.

Transfection of HEC-1B with pRC-2

a 0.8pg pRC-2 and 2pl LF

b 0.8pg pRC-2 and 2pl LF2K

KM U Mi

c 0.8jg pEGFP and 2pl LF2K

Figure 39

Transfection of HEC-1B cell line with a 0.8jg pRC-2 and 2pl LF and b 0.8pg pRC-2 and 2pl LF2K. Filters used were 1 DAPI for the nucleus and 2 FITC for EGFP. Composite images are shown in 3. Magnification x400.

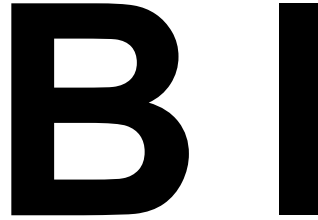
From the previous images it was noted that the green pRC-2 signals showed a distinctly cytoplasmic distribution in both cell lines, with prominent delineation around the nuclear membrane or perinuclear region. Cytoplasmic localisation of PTEN has been reported in endometrial stromal cells, whilst P-PTEN was observed in the nucleus (Guzeloglu-Kayisli, et al 2003). Varying amounts of protein and 'speckles' appeared in the approximate location as the nucleus, as described in neuronal cells by Lachyankar, et al (2000). Overall expression levels varied widely within the visible cells. The patterns of distribution and expression levels were comparable between pRC-2 and pEGFP. Of the two transfection reagents used, Lipofectamine 2000 (LF2K) gave a higher yield of transfected cells (Figure 38b & 39b) and was therefore selected to perform further investigation.

3.2.9.3 Optimisation of Transfection

HEC-1B and Ishikawa cells were seeded onto coverslips coated in polylysine, (Chapter 2, Section 2.3.12), and were observed to exhibit greater adherence as fewer cells became detached during the fixing process. Transfection rates were scored visually from several experiments performed independently with duplicate samples. Figures 40 to 43 show transfection levels using 2 μ l LF2K, and 0.6-1.0 μ g of pRC-2 for these cell lines at 200- and 400-times magnification.

Optimisation of HEC-1B Cell Line Transfection

a 0.6pg pRC-2 and 2pl LF2k x200



ai 0.6pg pRC-2 and 2pl LF2k x400

b 0.7pg pRC-2 and 2pl LF2k x200



bi 0.7pg pRC-2 and 2pl LF2k x400

Figure 40

Transfection of HEC-1B cell line grown on polylysine with 0.6 to 1.0pg pRC-2 and 2pl LF2K, a-d. Filters used were 1 DAPI for the nucleus and 2 FITC for EGFP. Composite images are shown in 3. Magnification x200 & x400.

c 0.8pg pRC-2 and 2pl LF2k x200

ci 0.8pg pRC-2 and 2pl LF2k x400

m

m

d 1.0pg pRC-2 and 2pl LF2k x200

Figure 40 (Continued)

Transfection of HEC-1B cell line grown on polylysine with 0.6 to 1.0pg pRC-2 and 2pl LF2K, a-d. Filters used were 1 DAPI for the nucleus and 2 FITC for EGFP. Composite images are shown in 3. Magnification x200 & x400.

Figure 41

High power images of HEC-1B cell line grown on polylysine with 0.8 pg pRC-2 and 2pl LF2K. Magnification x1000.

Optimisation of Ishikawa Cell Line Transfection

1

2

3

a 0.6pg pRC-2 and 2pl LF2k x200

ai 0.6ljg pRC-2 and 2pl LF2k x400

b 0.7pg pRC-2 and 2pl LF2k x200

c 0.8pg pRC-2 and 2pl LF2k x200

ci 0.8pg pRC-2 and 2pl LF2k x400

wmm

d 0.9pg pRC-2 and 2pl LF2k x200

e 1.Opg pRC-2 and 2pl LF2k x200

H k W T 1
■ i

" 7 b i J

ei 1.Opg pRC-2 and 2pl LF2k x400

Figure 42

Transfection of Ishikawa cell line grown on polylysine with 0.6 to 1.0pg pRC-2 and 2pl LF2K, a-ei. Filters used were 1 DAPI for the nucleus and 2 FITC for EGFP. Composite images are shown in 3. Magnification x200 & x400.

Figure 43

High power image of Ishikawa cell line grown on polylysine with 0.8 pg pRC-2 and 2pl LF2K. Magnification x1000.

The results for HEC-1B indicated that the best transfection rates of 50-70% were obtained using 0.8-1.0µg of pRC-2 (Fig 40 c-d). The majority of the transfected cells were seen to be expressing the PTEN protein at low levels, but a greater number of cells expressed the protein highly in proportion to increasing the amount of pRC-2 used.

The data obtained for Ishikawa cells showed that the best transfection rates of 30-50% were again obtained using 0.8-1.0µg of pRC-2 (Fig 42 c-ei). The majority of the transfected Ishikawa cells were seen to be expressing the GFP protein at low levels, but as observed with HEC-1B a greater number of cells expressed the protein highly in proportion to increasing the amount of pRC-2 used. Although Ishikawa cells do not express PTEN protein, the nuclei of transfected cells were observed undergoing division, and reconstitution of PTEN did not appear to cause apoptosis as nuclear fragmentation was not observed.

3.2.9.4 Stimulation of Transfected Cell Lines

Localisation of PTEN in HEC-1B and Ishikawa cell lines was investigated under stimulation using TGF-β1 or oestrogen, a hormone which influences cellular conditions in the endometrium. Oestrogen plays a major role in regulating the architecture of the endometrium during the menstrual cycle, and may therefore cause morphological changes in endometrial cells which could be linked to a change in PTEN protein localisation. Although TGF-β1 did not appear to modify PTEN protein levels in the experiments previously described here, it is worthwhile investigating whether the cytokine can change the localisation of the protein. Such changes in location within the cell may indicate sequestration to specific compartments which may have an

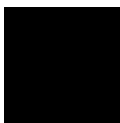
impact on the cellular function of PTEN in attenuating PI3K, and therefore growth regulation.

Briefly, cells were transfected with 0.8µg pRC-2 in the presence of 2µl LF2K and stimulated with the relevant compound as described in Chapter 2, Section 2.3.13. As cellular response to hormones and cytokines can be rapid, short incubation times of 1-2 hours were used. Longer incubations of up to 24 hours were employed to detect any delayed or long-lasting response.

3.2.9.4.1 Oestrogen stimulation of HEC-1B and Ishikawa cell lines

Concentrations of oestrogen (10 and 100nM) were chosen on the basis of published work (Campbell, et al 2001). Transiently transfected cells were stimulated with hormone and fixed for fluorescent microscopy after 2, 6 or 24 hours. These experiments were performed in triplicate on several separate occasions. Results for oestrogen stimulation of Ishikawa and HEC-1B cells lines are shown in Figures 44 to 47 and are representative of all the experiments.

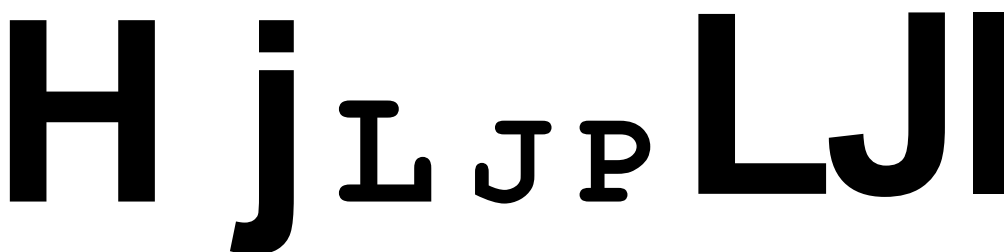
Oestrogen Stimulation of Transfected Ishikawa Cell Line



a 10nM oestrogen, 2 hrs x200

b 10nM oestrogen, 24 hrs x200

c 100nM oestrogen, 2 hrs x200



d 100nm oestrogen, 24hrs x200

Figure 44

Stimulation of transfected Ishikawa cell line with 10-100nM oestrogen for 2 and 24 hours a-d. Filters used were 1 DAPI for the nucleus and 2 FITC for EGFP. Composite images are shown in 3. Magnification x200

1

2

3

100nm oestrogen, 24hrs

Figure 45

High power image of transfected Ishikawa cell stimulated with 100nM oestrogen for 24 hrs. Filters used were 1 DAPI for the nucleus and 2 FITC for EGFP. Composite images are shown in 3. Magnification x1000.

Oestrogen Stimulation of Transfected HEC-1B Cell Line

a 10nM oestrogen, 2hrs x200

b 10nM oestrogen, 6hrs x200

I J

c 10nM oestrogen, 24hrs x200

Figure 46

Stimulation of transfected HEC-1B cell line with 10-100nM oestrogen for 2 and 24 hours a-c. Filters used were 1 DAPI for the nucleus and 2 FITC for EGFP. Composite images are shown in 3. Magnification x200

d 100nM oestrogen, 2hrs x200

Ei

e 100nM oestrogen, 6 hrs x200



f 100nM oestrogen, 24hrs x200

Figure 46 (Continued)

Stimulation of transfected HEC-1B cell line with 10-100nM oestrogen for 2,6 and 24 hours d-f. Filters used were 1 DAPI for the nucleus and 2 FITC for EGFP. Composite images are shown in 3. Magnification x200.

The results shown in Figure 44 and 45 indicated no discernable change in the distribution of PTEN in Ishikawa cells with the concentrations of hormone and incubation times used. Ishikawa cells are known to express functional oestrogen receptors (Nishida, 2002), but stimulation with this hormone appeared to have little effect on PTEN localisation. Cells from this experiment were very difficult to photograph compared to HEC-1B due to cloudiness on the coverslips which prevented good focussing of the microscope. This may represent morphological changes, eg rounding of the cells, but this experiment would need to be repeated using confocal microscopy to fully assess this observation.

The images presented in Figure 46 indicated no change in the overall distribution of PTEN in HEC-1B with the concentrations of hormone and incubation times used. As with the Ishikawa cell line, stimulation of transiently transfected HEC-1B cells appears to have little or no effect on the localisation of PTEN protein contributed by pRC-2.

3.2.9.4.2 TGF- β 1 Stimulation of HEC-1B cell line

Transiently transfected HEC-1B cells were stimulated with either 2ng/ml or 5ng/ml of cytokine, as these concentrations were previously used in the investigation of PTEN mRNA and protein expression. As for oestrogen, short incubation times of 1, 2 and 6 hours were used as cellular responses to cytokines can be rapid. Results for TGF- β 1 stimulation of the HEC-1B cells line are shown in Figure 47.

TGF-P1 Stimulation of Transfected HEC-1B Cell Line

a 2ng TGF-pi, 1hr x200

b 2ng TGF-p1, 2hr x200

B j

c 2ng TGF-p1,6hrs x200

Figure 47

Stimulation of transfected HEC-1B cell line with 2 and 5ng/ml TGF-p1 for 1,2 and 6 hours a-c. Filters used were 1 DAPI for the nucleus and 2 FITC for EGFP. Composite images are shown in 3. Magnification x200.

M i

d 5ng TGF-p1, 1hr x200

% >

e 5ng TGF-p1, 2hrs x200

T A

f 5ng TGF-p1, 6hrs x200

Figure 47 (Continued)

Stimulation of transfected HEC-1B cell line with 2 and 5ng/ml TGF-p1 for 1,2 and 6 hours d-f. Filters used were 1 DAPI for the nucleus and 2 FITC for EGFP. Composite images are shown in 3. Magnification x200.

Figure 47 shows the results of stimulating transiently transfected HEC-1B cells with TGF- β 1. No discernable change in the localisation of PTEN encoded by pRC-2 was observed with the concentrations of hormone and incubation times used.

Although no changes in the distribution of PTEN were detected in the stimulation experiments performed, it is possible that if any response occurred it was too rapid for observation or so slow that it occurred outside of the 24 hour maximum incubation time used.

3.2.9.5 Localisation of pRC-2 to Subcellular compartments

A comparison of the localisation of PTEN encoded by pRC-2 with colligin (ER marker) and mannosidase II (golgi marker) was performed to determine any co-localisation of PTEN to the ER or golgi. Localisation to specific regions of the cell may indicate partitioning of PTEN and have implications for the phosphatases' ability to gain access to its substrates. Additionally, close association with the ER may be important for the nuclear translocation of PTEN, the mechanism of which has not yet been described.

As described in Chapter 2, Section 2.3.14, transiently transfected HEC-1B and Ishikawa cell lines were probed with antibodies against colligin (Hsp47), an ER-resident protein, or golgi-specific anti-mannosidase II. Any overlap between the green signal from pRC-2 and the red signal from colligin or mannosidase II show as a yellow colour on the merged images.

Results for localisation with colligin are presented in Figures 48a/b and 49a/b for HEC-1B and Ishikawa cell lines respectively. Localisation studies with mannosidase II are shown in Figures 50 a/b and 51a/b for HEC-1B and Ishikawa cell lines respectively, and represent several separate experiments.

Localisation of Colligin & pRC-2 in HEC-1B Cell Line

»

Figure 48a

Anti-colligin staining of HEC-1B transiently transfected with pRC-2. Filters used were 1 FITC for EGFP, 2 Texas red for AlexaFluor 594 (Colligin), 3 DAPI for the nucleus. Composite image shown in 4.

Magnification x400.

Figure 48b

Anti-colligin staining of HEC-1B transiently transfected with pRC-2. Filters used were 1 FITC for EGFP,2 Texas red for AlexaFluor 594 (Colligin),3 DAPI for the nucleus. Composite image shown in 4.

Magnification x400.

Localisation of Colligin & pRC-2 in Ishikawa Cell Line

3

Figure 49a

Anti-colligin staining of Ishikawa transiently transfected with pRC-2. Filters used were 1 FITC for EGFP, 2 Texas red for AlexaFluor 594 (Colligin), 3 DAPI for the nucleus. Composite images are shown in 4. Magnification x400.

Localisation of Colligin & pRC-2 in Ishikawa Cell Line

3

Figure 49a

Anti-colligin staining of Ishikawa transiently transfected with pRC-2. Filters used were 1 FITC for EGFP,2 Texas red for AlexaFluor 594 (Colligin),3 DAPI for the nucleus. Composite images are shown in 4. Magnification x400.

1

2

Figure 49b

Anti-colligin staining of Ishikawa transiently transfected with pRC-2. Filters used were 1 FITC for EGFP,2 Texas red for AlexaFluor 594 (Colligin),3 DAPI for the nucleus. Composite images are shown in 4. Magnification x400.

Localisation of Mannosidase II & pRC-2 in HEC-1B Cell Line

2

3

Figure 50a

Anti-Mannosidase II staining of HEC-1B transiently transfected with pRC-2.

Filters used were 1 FITC for EGFP,2 Texas red for AlexaFluor 594 (Mannosidase II),3 DAPI for the nucleus. Composite images are shown in 4.

Magnification x400.

1

2

Figure 50b

Anti-Mannosidase II staining of HEC-1B transiently transfected with pRC-2.

**Filters used were 1 FITC for EGFP,2 Texas red for AlexaFluor 594
(Mannosidase II),3 DAPI for the nucleus. Composite images are shown in 4.**

Magnification x400.

Localisation of Mannosidase II & pRC-2 in Ishikawa Cell Line

3

Figure 51a

Anti-Mannosidase II staining of Ishikawa transiently transfected with pRC-2.

Filters used were 1 FITC for EGFP, 2 Texas red for AlexaFluor 594 (Mannosidase II), 3 DAPI for the nucleus. Composite images are shown in 4.

Magnification x400.

1

2

Figure 51b

Anti-Mannosidase II staining of Ishikawa transiently transfected with pRC-2.

**Filters used were 1 FITC for EGFP,2 Texas red for AlexaFluor 594
(Mannosidase II),3 DAPI for the nucleus. Composite images are shown in 4.**

Magnification x400.

As the images in Figures 48a and b show, there was some degree of overlap between PTEN and colligin giving a yellow colour on the composite images. This suggests that a proportion of the PTEN fusion protein was present in the ER, and that the presence of the GFP moiety did not prevent entry into this compartment. Additional signals derived from the FITC filter once demonstrate the presence of a small amount of exogenous PTEN in the cytoplasm and nuclear region.

Figures 50a and b show that there was no discernable overlap between pRC-2-derived PTEN and the golgi marker Mannosidase II in HEC-1B cells. The fusion protein was therefore not specifically localised to the golgi. However, the staining of the golgi was indistinct and diffuse particularly in transfected cells. The poor definition was possibly due to the polyclonal nature of the anti-Mannosidase antibody, which could be less specific than a monoclonal.

Figures 49a & b show there was some degree of overlap between pRC-2 and Colligin in Ishikawa cells, giving a yellow colour on the composite images. This suggests that at least some of the PTEN fusion protein was present in the ER, and that as observed in HEC-1B cells the presence of the GFP moiety did not exclude entry into this compartment. Additional signals derived from the FITC filter once again demonstrated the presence of exogenous PTEN in the cytoplasm and nuclear region.

Figures 51a and b show that there was very little overlap between pRC-2-derived PTEN and the golgi marker Mannosidase II in Ishikawa cells. The fusion protein was therefore not specifically localised to the golgi, as there was a partial overlap of colours in only a small proportion of cells. The

staining of the golgi was again indistinct and diffuse particularly in transfected cells, although somewhat clearer definition of the golgi was observed in the Ishikawa cell line.

3.2.9.6 Co-localisation of exogenous PTEN to endogenous PTEN in HEC-1B

To determine whether endogenous PTEN in transfected cells would demonstrate a different staining pattern to exogenous PTEN-EGFP, transfected HEC-1B cells were incubated with anti-PTEN primary (BD Biosciences) and AlexaFluor 594-labelled secondary antibodies (Chapter 2, Section 2.3.14). The images obtained are presented in Figures 52 a and b. In Figure 52 a low expression level of endogenous PTEN can be observed in non-transfected cells which has been detected by the anti-PTEN antibody and visualised under a Texas red filter. In contrast, the level of exogenous PTEN in transfected cells is significantly increased, suggesting that the endogenous cellular level of PTEN protein is fairly low.

From these images, a total overlap between the red and green images was observed, suggesting that both sources of PTEN localise to the same cellular regions. It should be noted, however, that if the anti-PTEN antibody is capable of binding to PTEN-GFP, then this experiment would not discriminate between the endogenous and exogenous forms of PTEN. The antibody's ability to bind to PTEN-GFP could be tested by Western blot against protein lysates from transfected Ishikawa cells which contain no endogenous PTEN protein.

Co-Localisation of PTEN and PTEN-EGFP in HEC-1B Cell Line

2

Figure 52a

Anti-PTEN staining of HEC-1B transiently transfected with pRC-2. Filters used were 1 FITC for EGFP, 2 Texas red for AlexaFluor 594 (PTEN), 3 DAPI for the nucleus. Composite images are shown in 4. Magnification x400.

Figure 52b

Anti-PTEN staining of HEC-1B transiently transfected with pRC-2. Filters used were 1 FITC for EGFP, 2 Texas red for AlexaFluor 594 (PTEN), 3 DAPI for the nucleus. Composite images are shown in 4. Magnification x1000

3.3 MUTATIONAL ANALYSIS OF PTEN EXONS 5 & 8 IN ENDOMETRIAL DNA SAMPLES BY SINGLE STRAND CONFORMATIONAL POLYMORPHISM ANALYSIS (SSCP)

Various studies have been conducted into the mutation status of genomic PTEN in fresh tumour tissue of diverse origin, but little work has been published using archival material (paraffin fixed and frozen samples which may have been stored for several years). This is mainly due to technical difficulties associated with retrieving DNA from paraffin embedded tissue sections. Extracted DNA can be highly fragmented and overall yields low, and the isolation of DNA from only one cell type is difficult if the section contains a mixture of cells.

Due to the availability of a large repository of archival endometrial carcinomas, complete with histologically normal cervical sections, a study of PTEN mutations was performed for the 43 patients available (a total of 127 samples). Because a histologically normal sample was available for each accompanying tumour specimen, any mutations in PTEN could be compared between normal and tumour DNA, thus such changes could be described as early or late events. Published results for fresh tumours described a range of mutations which were mainly clustered in exons 5 and 8 of PTEN, and therefore prompted my focus on these two exons. The electrophoretic technique of SSCP was chosen as a relatively simple and rapid method of analyzing the 127 samples for both exons, a total of 508 PCR samples.

SSCP is an electrophoretic method which is capable of detecting single base changes in a population of small DNA strands which are otherwise identical in sequence to a known sequenced control. Single stranded DNA forms

stable intramolecular secondary structures in solution, therefore differences in base sequence will manifest as differences in single-strand conformations. This technique distinguishes between these conformations by virtue of differences in DNA mobility through the gel. When compared to the control, sequence variations can be observed as 'bandshifts' which display a change in migration pattern. Controls used in SSCP analysis were derived from peripheral blood lymphocytes, in which mutations in PTEN are uncommon (Dahia, et al 1999). These samples were donated by members of staff and denoted as rDNA, gDNA, C1 and SJ1. Sequencing of these controls was performed to verify a wild-type PTEN sequence.

Small PCR fragments of <250bp in length are required for a good detection rate using this method. For this reason, Exons 5 and 8 were 'split' into two overlapping PCR reactions using primer pairs AB and CD, which contain a small region of overlap to amplify the entirety of each exon in these two reactions.

3.3.1 Preparation of DNA Samples for SSCP Analysis

3.3.1.1 Extraction of human DNA from whole blood and archival material

Extraction of DNA from whole blood and archival material was performed as described in Chapter 2, Section 2.3.2 and stored at -70°C prior to use in PCR reactions.

3.3.1.2 PCR amplification of Patient & Control DNA samples for SSCP Analysis

3.3.1.2.1 PCR amplification of samples for Exon 5

PCR products for Exons 5AB and 5CD were obtained for each patient sample from Dr K Feeley, University of Sheffield UK. PCR was performed using the conditions described in Chapter 2, Section 2.4.2 using the PTEN I programme (Appendix V). The presence of PCR products for 5AB (137bp) and 5CD (186bp) were confirmed by 15% PAGE.

Due to a very low yield of product amplified for 5AB in the PCRs sent to me, aliquots of all remaining patient DNA samples were requested from Dr Feeley. All patient DNA samples available, and controls, were therefore subjected to repeated PCR for 5AB using the RIS2-2 cycle (Appendix III) with the conditions described previously. PCRs were analysed by agarose gel electrophoresis, and the majority of samples exhibited an improved yield of the 5AB fragment when compared to PCR products generated by the PTEN I programme. Examples of these PCRs are illustrated in Figure 53 which shows the typical yield of some of the original samples, and Figure 54 which shows the improved yield using the RIS2-2 PCR cycle.

PCR samples provided for exon 5CD showed good amplification in the majority of patient DNA samples; an example is shown in Figure 55. Control 5CD DNA (RDNA) is shown in Figure 56.

PCR of Patient and Control samples for Exon 5AB

92 93 227 228 229 224 225 226 220 222

Figure 53 15% PAGE of 5AB patient samples 92-222, 10pl/ lane, PTEN I cycle.

M - 1 2 3

Figure 54 5AB PCR of 1: control rDNA 2: patient 49 3: patient 52x, 10pl/lane, RIS2.2 cycle. Dash indicates PCR control, M: 100bp ladder.

PCR of Patient and Control samples for Exon 5CD

Figure 55 15% PAGE of 5CD PCR for patient samples 84-93, 10pl/ lane, PTEN I cycle.

M - 1

Figure 56 5CD PCR of 1: control rDNA, 10pl per lane, PTEN I cycle. Dash indicates PCR control.
M:100bp ladder.

3.3.1.2.2 PCR amplification of samples for Exon 8

PCR products for Exons 8AB were obtained for each patient sample from Dr K Feeley, as described, and the presence of the 8AB fragment (188bp) confirmed by 15% PAGE. For Exon 8CD, PCR was performed on available patient DNA using the PTEN I cycle (Chapter 2, Section 2.4.2). The presence of the 8CD (245bp) fragment was confirmed by agarose gel electrophoresis. For exon 8AB, all patient DNA samples showed good amplification; an example is shown in Figure 57. Control DNA amplified using PTEN I cycle is shown in Figure 58. PCR of all patient DNA samples available for exon 8CD was performed as previously described. Control DNA is shown in Figure 59.

PCR of Patient and Control samples for Exon 8 AB

Figure 57 15% PAGE of 8AB PCR for patient samples 84-92, 10pl/ lane, PTEN 1 cycle. M= 100bp ladder.

M - 1 2

Figure 58 8AB PCR of controls 1: RDNA, 2: gDNA, 10_{pl} per lane. Dash indicates PCR control

PCR of Control samples for Exon 8 CD

M 1

Figure 59 8CD PCR of 1: Control gDNA, 10pl. Dash indicates PCR control M= 100bp

3.3.2 Preparation of Control PCR products for sequencing

To provide normal sequence controls for SSCP, PCR was performed using DNA derived from peripheral blood donated by four staff members. PTEN mutations are very rare in white blood cells, so these samples were likely to have normal sequences from which to compare patient DNA. Staff control DNA (rDNA, gDNA, C1 and SJ1) to be used as controls was purified as described in Chapter 2, Section 2.4.3, following PCR with AB and CD primer pairs for both exons as previously described for patient DNA (Section 2.4.2). Sequence information was obtained from Lark as electropherograms (see Figures 60 to 68). Adequate sequences were obtained for exon 5AB, 5CD and 8AB, for either g- or r-DNA, C1 and SJ1 control samples. The sequences showed a high degree of sequence homology to PTEN cDNA with few ambiguous bases. These samples were regarded as suitable controls for these exon fragments. Sequence data for the 8CD control was not obtained due to poor read quality after several attempts, but was still used on the relevant gels.

No electropherogram for gDNA 5A

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Figure 63 rDNA control 50D sequences

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Figure 65 rDNA control 8AB sequences

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Figure 66 C1 control 8AB sequences

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Figure 67 S11 control DNA 8AB

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offering from published sequence. Dotted line indicates the point at which the read degenerated.

3.3.3 SSCP Analysis

PCR products were initially separated using a minigel (8 x 10 cm) system. This method, however, was revised due to poor band migration. Increased band separation and detection sensitivity was observed using an 18x24 cm large gel format, therefore this method was selected to analyse the PCR products for all exon fragments. SSCP was performed as described in Chapter 2, Section 2.4.4 using 0.5 - 0.6% MDE/10% glycerol gels running overnight at 4°C to avoid heat generation. Bands were detected initially by manual silver staining (Section 3.3.3.2), which gave a high background and poor band definition, and later with a silver staining kit as described in Chapter 2, Section 2.4.4.2.

Results of SSCP are shown in Figures 69 and 70, for exon 5 (Section 3.3.3.1-2) and Figures 76 and 77 for exon 8 (Section 3.3.3.3-4). Samples were carefully compared to the band migration pattern of one or more of the controls (rDNA, gDNA, C1 or SJ1). Samples showing band shifts were noted and re-run on separate gels to confirm the presence of the aberrant bands (highlighted in red), where sufficient sample was available. The results for each exon were tabulated to include histological details for each sample, where available (Tables 19 to 22).

3.3.3.1 Exon 5AB SSCP Results

Fig 69a

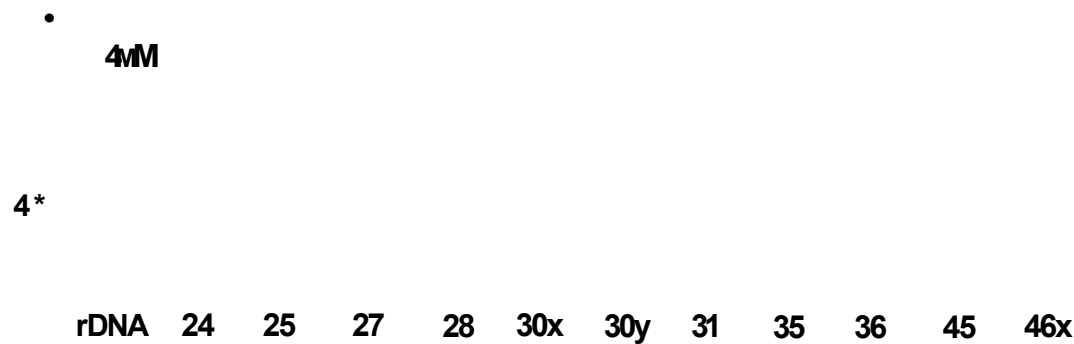


Fig 69b

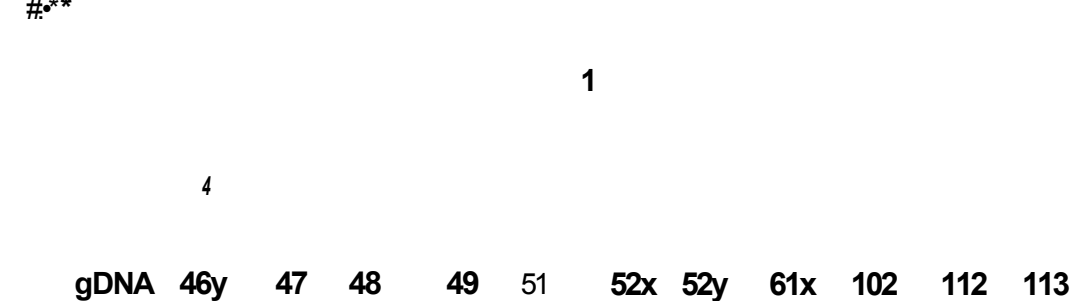


Fig 69c

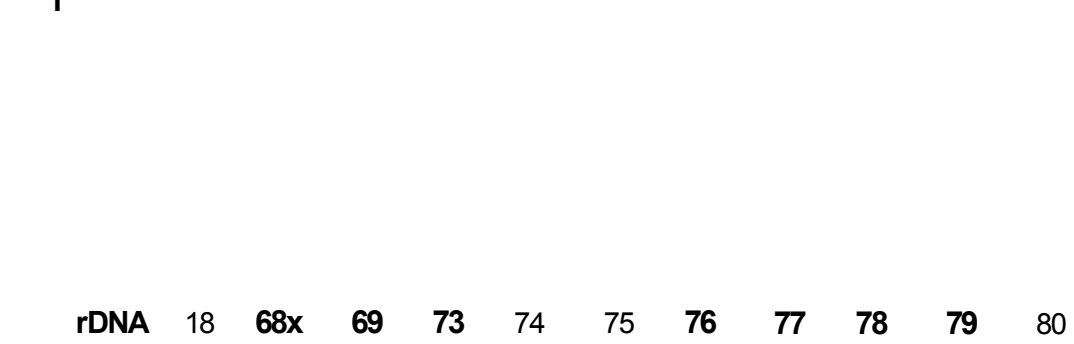


Fig 69d

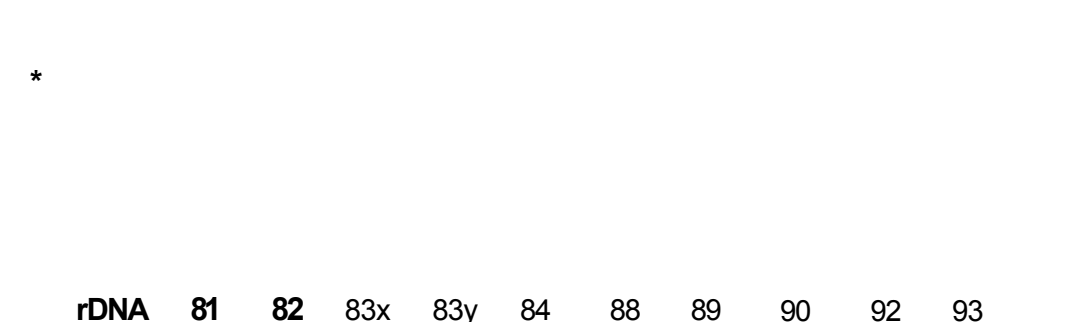


Figure 69 a-d SSCP results for exon 5AB. Samples with bandshifts are numbered in red. Controls rDNA and/or gDNA are included with 100bp marker on each gel (lane 1).

Fig 69e

rDNA 114 134 135 137 138 151 152 153 159 160 gDNA

rDNA 161 162 219 220 222 224 226 227 228 229 gDNA

Fig 69g

rDNA 207 208 209 210 211 212 214x 216 217 218 gDNA

Fig 69 e-g SSCP results for exon 5AB

Samples with bandshifts are numbered in red. Controls rDNA and/or gDNA are included with 100bp marker on each gel (Lane 1).

| Table 19 Bandshift Summary Exon 5AB | | | |
|--|-------------|---------------|------------------|
| Sample | ID | Tissue | Histology |
| 18 | 95/23231e | Endometrium | AH? |
| 51 | 94/18507c | Endometrium | AH? |
| 74 | 97/12804 ln | Endometrium | Com H |
| 83x | 97/21010 jx | Endometrium | AdCa 2 & AH |
| 83y | 97/21010 jy | Endometrium | AdCa 2 & AH |
| 84 | 97/21010 a | Cervix | Normal |
| 88 | 98/4189 2a | Cervix | Normal |
| 89 | 98/4189 2d | Endometrium | AdCa 2 |
| 90 | 98/4189 2c | Endometrium | AdCa 2 & AH |
| 92 | 98/11642 h | Endometrium | AdCa 3 |
| 93 | 98/11642 g | Endometrium | AH? |
| 152 | 94/11672 c | Endometrium | H |
| 153 | 94/11672 b | Cervix | Normal |
| 159 | 94/20749 c | Endometrium | H |
| 160 | 95/6941 c | Endometrium | H |
| 208 | 97/3404 f | Endometrium | Marginal |
| 209 | 94/4094 f | Endometrium | - |

Table 19 Bandshift Summary for Exon 5AB showing samples in which aberrant bands were detected. Where available, tissue and histological description are included. **H:** hyperplasia, **AH:** atypical hyperplasia, **Com H:** complex hyperplasia, **AdCa (x):** adenocarcinoma (grade), **?:** histological grouping unclear.

3.3.3.2 Exon 5CD SSCP Results

Fig 70a

rDNA 18 18a 20 21 22 24 25 27 28 134 135

rDNA 30x 30y 31 67 68x 68y 69 112 113 114

rDNA 35 36 45

Fig 70d

rDNA 35 36 45 - 46x 46y 47 48 49 C1

Figure 60 a-d SSCP results for exon 5CD. Samples with bandshifts are numbered in red. Controls rDNA and/or gDNA are included with 100bp marker on each gel.

| rDNA | 50 | 60 | 61x | 61y | 195 | 196 | C1 |
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|------|----|----|-----|-----|-----|-----|----|

Fig 70f

73 74 75 76 77 78 79 80 81 82

rDNA 83x 83y 87 88 89 90 91 92 93 rDNA

70h

Figure 60 e-h SSCP results for exon 5CD. Samples with bandshifts are numbered in red. Controls rDNA and/or gDNA are included with 100bp marker on each gel (lane 1).

rDNA 160 161 162 163 166 167 168 169 170 171 rDNA

rDNA 205 206 207 208 209 210 211 212 214 215 rDNA

rDNA 216 217 218 219 220 221 222 rDNA (8AB)

rDNA 224 225 226 227 228 229 rDNA (8AB)

Figure 60 i-l SSCP results for exon 5CD. Samples with bandshifts are numbered in red. Controls rDNA and/or gDNA are included with 100bp marker on each gel (lane 1).

rDNA 153 154 172 173 174 175 176 177 178 rDNA

Fig 70n

rDNA 179 180 185 186 189 190 191 192 rDNA

Fig 70o

rDNA 20 21 22 67 68x 68y 69 164 165 191 192

Fig 60 m-o SSCP results for exon 5CD. Samples with bandshifts are numbered in red. Controls rDNA and/or gDNA are included with 100bp marker on each gel (lane 1).

| Table 20 Bandshift Summary Exon 5CD | | | |
|-------------------------------------|--------------|-------------|---------------|
| Sample | ID | Tissue | Histology |
| 22 | 96/11307 h | Endometrium | - |
| 46x | 94/5212 lbx | Endometrium | AdCa 3 + AH? |
| 61x | 95/22466 5fx | Endometrium | IECa / Sev AH |
| 61y | 95/22466 5fy | Endometrium | IECa / Sev AH |
| 67 | 97/9145 3b | Cervix | Normal |
| 68y | 97/9145 32y | Endometrium | AdCa 2 |
| 69 | 97/9145 lb3 | Endometrium | AH / IECa |
| 165 | 94/21552 3a | Cervix | Normal |
| 192 | 97/3362 e | Endometrium | - |

Table 22 Bandshift Summary for Exon 5CD showing samples in which aberrant bands were detected. Where available, tissue and histological description are included. **AH:** atypical hyperplasia, **Com H:** complex hyperplasia, **Sev AH:** severe atypical hyperplasia, **AdCa (x):** adenocarcinoma (grade), **IECa:** intraepithelial carcinoma, **?:** histological grouping unclear.

Exon 5 Summary

SSCP separation for the 5AB exon fragment was good, however interpretation of the bands was difficult due to multiple additional bands observed in each sample. Patient samples could not be sequenced due to inadequate read quality after several attempts.

Figures 70a-j show gels developed by manual silver staining. Sensitivity was very low and background staining high for the majority of gels. Figures 70k-o demonstrate the improvement of gels stained with the PlusOne silver staining kit (Chapter 2, Section 2.4.4.2).

With regard to exon 5CD, sequencing was performed on sample 46x, which exhibited a bandshift, and also samples 45, 46y and 47 obtained from the same patient. Sequence data was successfully obtained (Figures 71 to 74) but the readings were incomplete, containing ambiguous bases and low signal strength, especially with the 5C primer.

The readable portions of data were aligned with the PTEN cDNA sequence for comparison (Figure 75). No conclusions as to the success of bandshift detection could be made from the sequence data obtained, due to the high number of ambiguous bases or base changes.

Overall figures for the incidence of bandshifts in exon 5 were as follows. For exon 5AB, 26% (11/43) of patients demonstrated bandshifts from a corresponding total of 13% (17/127) of all samples. For exon 5CD, 14% (6/43) of patients demonstrated bandshifts from a corresponding total of 7% (9/127) of all samples. The total bandshift detection rate for exon 5 was 40% (17/43) of patients, from 20% (26/127) of the samples used.

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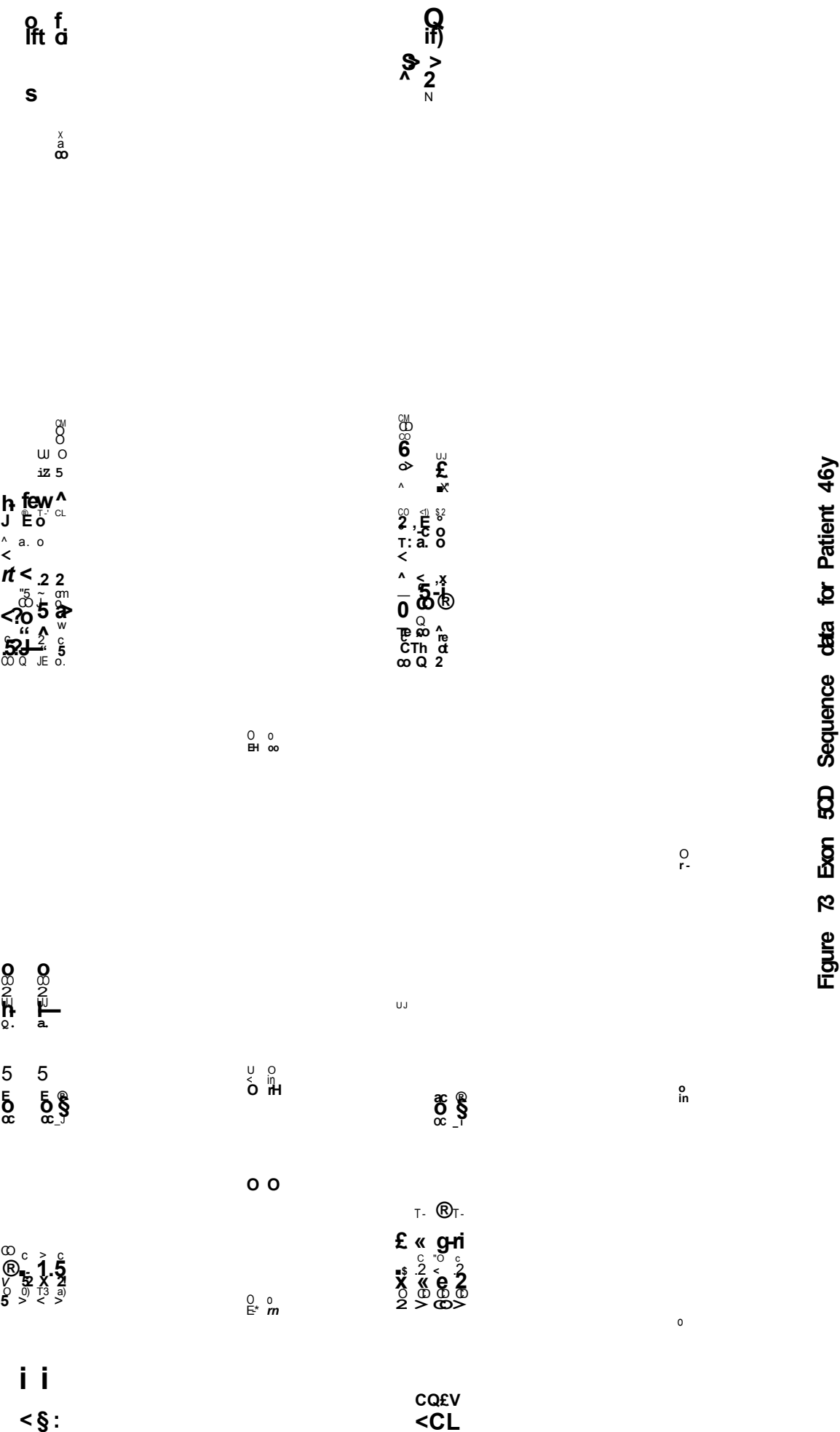
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Figure 71 Exon 50D Sequence data for Patient 45



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Fig 74 Exon 50D Sequence data for Patient 47

3.3.3.3 Exon 8AB SSCP Results

Fig 76a

rDNA 30x 30y 31 35 36 45 46x 46y 47 rDNA

Fig 76b

f A

mm

rDNA 48 49 50 51 52 60 61x 61y rDNA

Fig 76c

* * •*m*

rDNA 67 68x 68y 73 74 75 76 SJ1 C1

Fig 76d

rDNA 77 78 79 80 81 82 83x 83y 84 SJ1

Figure 76 a-d SSCP results for exon 8AB Samples with bandshifts are numbered in red. Controls rDNA and/or C1 and SJ1 are included with 100bp marker on each gel (lane 1).

| | | | | | | | | | | |
|----------|----|----------|----|-----------------------|----------|----------|----------|----------|----|------|
| | % | <i>m</i> | | | <i>m</i> | <i>m</i> | <i>m</i> | <i>m</i> | | |
| | | <i>m</i> | | <i>m_{//}</i> | | <i>m</i> | | | | |
| <i>t</i> | | | | | | | | | | |
| rDNA | 88 | 89 | 90 | 91 | 92 | 93 | 101 | 102 | C1 | rDNA |

| rDNA | 112 | 113 | 114 | 134 | 135 | 136 | 137 | 138 | SJ1 | rDNA |
|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|

| rDNA | 151 | 152 | 153 | 154 | 158 | 159 | 160 | 161 | 162 | 163 | C1 |
|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|
|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|

«*

Figure 76 e-h SSCP results for exon 8AB Samples with bandshifts are numbered in red. Controls rDNA and/or C1 and SJ1 are included with 100bp marker on each gel (lane 1).

Fig 76i

rDNA 174 175 176 177 178 179 180 185 186 C1

Fig 76j

rDNA 187 188 189 190 191 192 193 194 195 196 SJ1

Fig 76k

rDNA 200 201 202 203 204 205 206 207 - 208 C1

Fig 76l

■mm

rDNA 209 210 211 212 214 215 216 217 218 219 SJ1

Fig 76m

rDNA 220 221 222 224 225 226 227 228 229 C1

Figure 76 i-m SSCP results for exon 8AB Samples with bandshifts are numbered in red. Controls rDNA and/or C1 and SJ1 are included with 100bp marker on each gel (lane 1).

Fig 76n

rDNA 18 18a 24 25 50 51 52 60 61 x 61y SJ1

Fig 76o

mmm

rDNA 35 36 45 46x 46y 47 151 152 166 167

Figure 76 n-o SSCP results for exon 8AB. Samples with bandshifts are numbered in red. Controls rDNA and/or C1 and SJ1 are included with 100bp marker on each gel (lane 1).

| Table 21 Bandshift Summary Exon 8AB | | | |
|-------------------------------------|--------------|-------------|----------------|
| Sample | ID | Tissue | Histology |
| 25 | 96/17667 c | Endometrium | Com H |
| 35 | 97/23720 1a | Cervix | Normal |
| 36 | 97/23720 1p | Endometrium | AH (B'gr) |
| 47 | 94/5212 1j | Cervix | - |
| 48 | 98/1372 1a | Cervix | Normal |
| 49 | 98/1372 1d | Endometrium | AH |
| 50 | 94/18507 a | Cervix | Normal |
| 51 | 94/18507 c | Endometrium | AH? |
| 52 | 94/18507 f | Endometrium | Ad Ca 3 |
| 60 | 95/22466 5c | Cervix | Normal |
| 61x | 95/22466 5fx | Endometrium | IE Ca / Sev AH |
| 61y | 95/22466 5fy | Endometrium | IE Ca / Sev AH |
| 83y | 97/21010 j | Endometrium | AdCa2 + Ah |
| 84 | 97/21010 a | Cervix | Normal |
| 92 | 98/11642 h | Endometrium | Ad Ca 3 |
| 194 | 98/10452 j | Endometrium | - |

Table 21 Bandshift Summary for Exon 8 AB showing samples in which aberrant bands were detected. Where available, tissue and histological description are included. **AH**: atypical hyperplasia, **Com H**: complex hyperplasia, **Sev AH**: severe atypical hyperplasia, **IECa**: intraepithelial carcinoma, **AdCa (x)**: adenocarcinoma (grade), **?**: histological grouping unclear.

3.3.3.4 Exon 8CD SSCP Results

Fig 77a

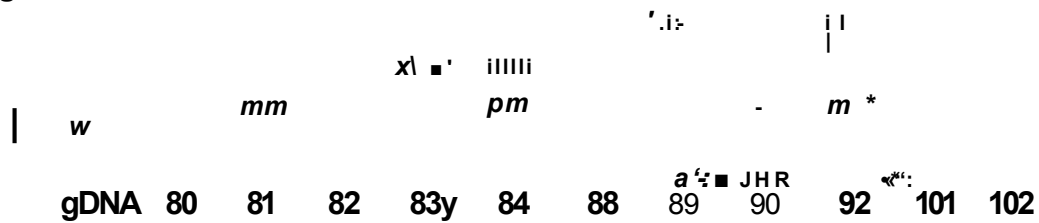


Fig 77b

*

gDNA 20 21 27 28 48 49 81 82 83y 84 88

Fig 77c

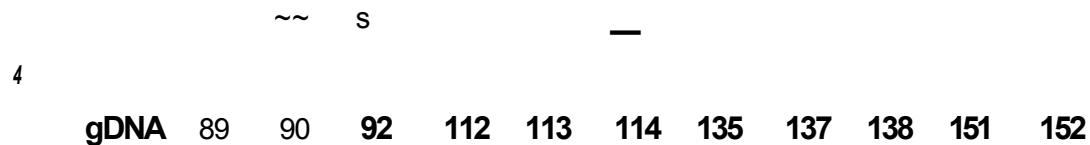


Fig 77d

gDNA 153 159 160 161 162 168 169 174 175 176 177

Fig 77e____

gDNA 167 171 172 179 180 185 186 188

Figure 77 a-e SSCP results for exon 8CD. Samples with bandshifts are numbered in red. Control gDNA is included with 100bp marker on each gel (lane 1).

Fig 77f

b 3 |MIS\$ -s ' 4

gDNA 189 190 192 193 194 195 196 202 52y 61x 61y

Fig 77g

◆

gDNA 67 68x 73 75 79 80 81 102 134 165 166

Figure 77 f-g SSCP results for exon 8CD. Samples with bandshifts are numbered in red. Control gDNA is included with 100bp marker on each gel (lane 1).

Table 22 Bandshift Summary Exon 8CD

| Sample | ID | Tissue | Histology |
|--------|------------|-------------|-------------|
| 89 | 98/4189 2d | Endometrium | AdCa 2 |
| 90 | 98/4189 2c | Endometrium | AdCa 2 & AH |

Table 22 Bandshift Summary for Exon 8 CD showing samples in which aberrant bands were detected. Where available, tissue and histological description are included. AH: atypical hyperplasia, AdCa (x): adenocarcinoma (grade).

Exon 8 Summary

Exon 8AB generated very good SSCP gels with clean, well-separated bands and very little background (Figures 76a-o). Sequences for patient samples sent for contained many ambiguous bases (data not shown) and were difficult to interpret. SSCP for exon 8CD fragments demonstrated much lower separation than for 8AB, and altering the gel composition had little effect in improving this (data not shown). Again, sequencing of patient samples 89 and 90 in which aberrant bands were detected contained many ambiguous bases (data not shown).

Figures for the incidence of bandshifts in exon 8 were as follows. For exon 8AB, 21% (9/43) of patients demonstrated bandshifts from a corresponding total of 12% (16/127) of all samples. For exon 8CD, 2% (1/43) of patients demonstrated bandshifts from a corresponding total of 2% (2/127) of all samples. The total bandshift detection rate for exon 8 was 23% (10/43) of patients, from 14% (18/127) of the samples used.

3.3.3.5 Patients with multiple bandshifts

Eight samples corresponding to five patients displaying bandshifts in more than one exon are shown in Table 23. Six samples carry an exon 5 AB or 8AB bandshift, with 4 samples carrying changes in both of these exon portions. This may reflect the sensitivity of the SSCP technique for the different exon portions. It is interesting to note that 6 out of the 8 samples (75%) displaying aberrations in two exons are histologically advanced lesions (adenocarcinomas). The higher grade of the samples (2 and 3) denotes increasing depth of invasion into the tissue underlying the endometrium.

| Table 23 Samples with Bandshifts in Multiple exons | | |
|---|------------------|--------------|
| Sample | Histology | Exons |
| 51 | AH? | 5AB & 8AB |
| 61x* | IECa / Sev AH | 5CD & 8AB |
| 61y* | IECa / Sev AH | 5CD & 8AB |
| 83y [†] | AdCa2 + AH | 5AB & 8AB |
| 84 [†] | Normal | 5AB & 8AB |
| 89 [§] | AdCa2 | 5AB & 8CD |
| 90 [§] | AdCa2 + AH | 5AB & 8CD |
| 92 | AdCa3 | 5AB + 8AB |

Table 23 Samples displaying bandshifts in multiple exons, with histological descriptions. Symbols indicate samples belonging to the same patient.

AH: atypical hyperplasia, **Sev AH:** severe atypical hyperplasia, **IECa:** intraepithelial carcinoma, **AdCa (x):** adenocarcinoma (grade), **?:** histological grouping unclear.

CHAPTER 4

DISCUSSION

4.1 Discussion of Results

Understanding the molecular mechanisms which underlie the initiation and progression of cancer is central to the development of novel therapeutic strategies. Since its discovery in 1997, the PTEN tumour suppressor has proven to be the first of an emerging species of lipid phosphatase involved in multiple cellular processes, which now includes SHIP and myotubularin phosphatase (Wishart & Dixon, 2002). The existence of PTEN and its several homologues, such as PTEN 2, TPTE and TPIP across the spectrum of eukaryotic organisms highlights the evolutionary importance of this enzyme. The central role of PTEN in the control of multiple signalling pathways, and its interaction with other cell-cycle proteins, have placed it amongst the hierarchy of tumour suppressor genes including pRb and p53. Recently, PTEN has proven to be much more than just a tumour suppressor by exhibiting a fundamental role in the development and organisation of tissues as diverse as neurones and immune cells.

Significantly, the high occurrence of PTEN mutation or loss of expression is a feature of certain cancers, including endometrial and prostate carcinomas, although the reasons for such tissue specificity are not yet fully understood. The exceptionally high incidence of PTEN aberrations in endometrial carcinomas (~80%) has provoked many studies, and unlike other somatic cancers, loss of PTEN is an early event in the development of EC. Clearly, PTEN has a fundamental role in endometrial pathology, and it is hoped that

by dissecting this role advances will eventually be made in the diagnosis and treatment of endometrial carcinoma.

The aims of this thesis were therefore to investigate the role of PTEN in endometrial carcinoma by three distinct approaches. These included analysis of the effect of TGF- β 1 on PTEN expression in EC cell lines, cloning of human PTEN cDNA and assessing sub-cellular localisation of the protein under various conditions, and the detection of novel mutations in EC archival DNA.

The effect of TGF- β 1 on PTEN expression in EC cell lines

TGF- β 1 has previously been shown to both up- and down-regulate PTEN mRNA or protein levels in various cell lines, in a manner which may indicate cell-line specificity, but this has not been previously reported in cells of endometrial origin. Compounds which up-regulate PTEN may represent future anti-cancer therapies, and therefore merit investigation.

For this study, cell lines were chosen which expressed wild-type (HEC-1B) or mutant (Ishikawa) PTEN protein. This permitted the study of the effects of the cytokine on growth and cellular morphology in the presence or absence of endogenous PTEN protein, in addition to modulation of PTEN mRNA and protein levels.

The results presented in Chapter 3, Section 1 indicated a modest up-regulation of PTEN mRNA expression in both HEC-1B and Ishikawa cell lines in response to TGF- β 1. Similar results have been reported by Lee, et al (1999) in the human monocytic leukaemia cell line U937, but this group did not describe a possible pathway for the data described. Serum deprivation

also appeared to have a small up-regulatory effect on PTEN mRNA expression. This could be accounted for by the lack of growth factors, which when present in the medium could suppress PTEN protein function, and possibly mRNA expression. Functionality of the TGF- β 1 receptor signalling to Smads in HEC-1B and ISK cell lines was confirmed by the time and dose-dependant up-regulation of the classically TGF- β 1-responsive gene PAI-1. Additionally, the effects of high cell density on increasing PTEN mRNA expression were ruled out, as plating HEC-1B cells at high density displayed no difference in PTEN expression to cells grown at a lower density (Chapter 3.1.5)

Analysis of PTEN and P-PTEN protein levels indicated very little change from the controls which were not stimulated with cytokine. This suggests that the effects of TGF- β 1 occurred mainly at the transcriptional level, or that the protein analysis methods used were not sensitive enough to detect subtle post-translational changes. It remains unclear, however, which of the three putative PTEN phosphorylation sites in the C-terminus (Ser380 and Thr382/383) are phosphorylated under differing cellular conditions. At the time that the work described here was performed, only an anti-P-PTEN (Ser380) antibody was commercially available. The use of a new anti-P-PTEN antibody (CST, UK), which detects phosphorylation of all three C-terminal sites (Ser380 and Thr382/383), may increase the sensitivity of the Western blot for P-PTEN.

Effects of TGF- β 1 on endometrial cell morphology agree with published data (Boyd & Kaufman, 1990 & Anzai, et al 1992) in that the cytokine increases cell volume and/or decreases proliferation of the HEC-1B and ISK cell lines.

The mechanisms underlying the regulatory effects of TGF- β 1 on PTEN are yet to be described. TGF- β 1 may exert growth suppression on these cell lines via several mechanisms described in Chapter 1. These may include cell cycle restriction via the transcription factor Sp1 which increases expression of the cyclin inhibitor p15^{INK}, increased FOXO activity, or by inhibition of c-myc. The presence of a Sp1 consensus site in the promoter region of PTEN has been noted by Sheng, et al (2002), and this may represent a pathway of transcriptional modulation by TGF- β 1.

Cloning of Human Full-Length PTEN and localisation of Transfected PTEN protein in Endometrial cell lines.

PTEN has been described as a cytoplasmic protein in the majority of cell lines and tissues, which would appear to correlate with a lack of nuclear location signal (NLS). However, nuclear PTEN has been reported in a number of studies and it is likely that other proteins are involved in its transport both to the nucleus and to various cellular compartments.

Other phosphatases have been observed to be shuttled between the nucleus and cytoplasm in synchrony with the cell cycle, including CDC25B (Davezac, et al 2000) and CDC14 (Li, et al 1997b). The discovery of a nuclear PI3,4,5P₃ cycle, which is still poorly understood, supports the theory that PTEN may have a role in the nucleus (Lachyankar, et al 2000). The extent to which nuclear PTEN responds to modulation by external stimuli, such as growth factors and hormones, is also not fully understood.

The results of transfection of pRC-2 into HEC-1B and Ishikawa cell lines are partially consistent with a recently published report by Ginn-Pease & Eng

(2003). This group used full-length PTEN cloned into both a vector without a fluorescent tag and also into the pEGFP-C1 vector, to transiently transfect the MCF-7 breast cancer cell line. The paper reported accumulation of transfected PTEN around the nuclear membrane and in the general location of the nucleus, which increased at G₀-G₁ and decreased during S-phase. Co-localisation of PTEN with the nuclear protein CDK2 during G1 was also observed. Additionally, the presence of PTEN in the nucleus was confirmed by Western blot of nuclear and cytoplasmic extracts prepared from the transfected cells. The presence of the N-terminal EGFP moiety did not reportedly affect PTEN entry into the nucleus or the cell cycle. The results shown in Chapter 3, Section 2 are strikingly similar to those of Ginn-Pease & Eng (2003) in that strong staining in the perinuclear and nuclear regions, but also ubiquitous cytoplasmic localisation, were observed in the endometrial cell lines. Some vault proteins can accumulate at the nuclear membrane adjacent to nuclear pores, and as PTEN can bind the major vault protein via its C2 domain (Yu, et al 2002) this may be a mechanism of transport into the nucleus. Other proteins, such as β -catenin, may bind directly to the nuclear pore to mediate their own transport, but in this case the mechanism is unknown (Suh & Gumbiner, 2003).

A further possibility proposed by Ginn-Pease and Eng is that PTEN may have a more rare form of NLS called a nuclear-cytoplasmic shuttling sequence, or a bipartite NLS. Such bipartite sequences have previously been reported in p53 (Liang & Clarke, 1999), and the CDK inhibitor p27^{KIP1} has been shown to require an interaction with Jab1/CSN5 for CRM1-mediated nuclear export (Tomoda, et al 2002).

A lack of detectable change in subcellular localisation of PTEN encoded by pRC-2 in endometrial cell lines in response to stimulation by oestrogen and TGF- β 1 was observed (Chapter 3, Section 2). With respect to oestrogen, Mutter, et al (2000) has described ubiquitous expression of PTEN in both the nucleus and cytoplasm of oestrogen-exposed dividing endometrial glandular and stromal cells. A further study by Guzeloglu-Kayisli, et al (2003) demonstrated a rapid and short-lived increase in total PTEN and nuclear P-PTEN in endometrial cells in response to oestrogen. This increase declined after 15 minutes and was absent in cells treated over longer periods (3 to 24 hours), suggesting that any increase in PTEN is extremely rapid and transitory. As the unstimulated distribution of PTEN-EGFP was observed to be both perinuclear/nuclear and cytoplasmic, oestrogen may not affect localisation of PTEN under the experimental conditions used. Cells stimulated with TGF- β 1 also demonstrated no change in exogenous PTEN localisation, suggesting that with consideration to the lack of endogenous PTEN increase shown by Western blot in Chapter 3, the cytokine does not modify levels of the protein.

Co-localisation of PTEN in endometrial cell lines with markers for the ER and Golgi shown in Chapter 3, Section 2 has not previously been described. Localisation of the murine testis-specific homologue of PTEN, called PTEN-2, to the golgi in transfected COS-7 cells has been demonstrated by Wu, et al (2001), who cloned PTEN-2 into pEGFP-N3. This localisation was shown to require the N-terminal domain of PTEN-2 containing four potential transmembrane domains, which may play a role in vesicular trafficking through the golgi network.

In the HEC-1B and ISK cell lines used in this study, very little co-localisation was observed between the golgi protein mannosidase II and PTEN. Although detection of the golgi with the non-commercial polyclonal antibody did not appear to be very specific, it is more likely that PTEN simply does not associate with the golgi in the same manner as testis-specific PTEN-2. Co-localisation of PTEN with the endoplasmic reticulum resident chaperone protein colligin (Hsp47) was detected in both endometrial cell lines as illustrated in Chapter 3, Section 2. As the ER is continuous with the outer nuclear membrane, it is possible that PTEN localises with colligin if it has been localised to the perinuclear region perhaps via one of the theoretical mechanisms previously described.

Recently, PTEN has been detected in lipid rafts within intestinal cells, which may affect PTEN-mediated attenuation of PI3K by specific localisation of the protein (Li, et al 2004). Lipid rafts and their stabilising caveolin proteins, are distinct micromembrane domains which have been implicated in signal transduction and can associate with many transduction elements, including receptor tyrosine kinases, PKC, G-proteins and Ras-MAPK components (Pike, L. J., 2003 & Smart, et al 1999). PTEN and other protein tyrosine phosphatases have previously been shown to form complexes with caveolin-1, which may regulate the function of these proteins (Caselli, et al 2002).

Mutation Analysis of PTEN Exons 5 & 8 in Archival Endometrial DNA by SSCP

A large variety of PTEN mutations have now been described in EC and other cancers. The SSCP technique used in this thesis was based on previous

reports (Rhei, et al 1997, Aveyard, et al 1999 & Cinti, et al 2000) in which a commercial mutation detection gel solution and silver staining kit were used to give superior separation and detection of bands. The results of SSCP analysis shown in Chapter 3, Section 3 indicate that excellent separation and staining was achieved for all samples with the exception of exon 8CD PCR products. From the total of total of 43 patients, 27 (63%) were found to have at least one sample containing a bandshift. This figure is in agreement with the published PTEN mutation rate in EC of 20-85%, which includes the earliest hyperplasias to the highest grade adenocarcinomas.

The SSCP technique, whilst being relatively simple, rapid, and up to 95% effective for some genes, is also extremely sensitive to changes in temperature and primer concentration (Hennessy, Teare & Ko, 1998). The latter of these variables may account in part for the poor separation of 8CD products. As stated in Chapter 3, sequencing data was difficult to obtain for the great majority of samples exhibiting bandshifts. The quality of DNA derived from archival material is notoriously fragile, and can generate not only low yields of PCR product but also reduce the specificity of the reaction and lead to spurious amplification. This is the main reason why archival DNA is not commonly used in such studies, where fresh tissue is preferred.

The PCR products for all samples appeared to consist of a single clean band, but insufficient PCR product was problematic in some samples, particularly in exon 5AB. Although non-specific amplicons were visually undetectable on the agarose gel, the presence of very small amounts of extraneous PCR product could be a cause for interference in sequencing as the technique is very sensitive to contamination.

A further explanation for the sequencing difficulties encountered is that more than one cell type was present in the tissue samples when DNA was extracted. Potentially, strands of DNA with slightly different sequences could therefore have been present in the samples prior to PCR, causing multiple amplicons of the same apparent size to be generated. As the tissue handling and DNA extraction were performed by our collaborators, this element of the study was essentially beyond my control.

Despite the associated technical hurdles, SSCP is still being used to effectively detect PTEN mutations and thereby increase our understanding of this gene in disease. One recent study has discovered three polymorphisms in PTEN from Japanese patients with type-2 diabetes, these being c.-9C→G in the 5' UTR of exon 1, IVS5-11T→G in intron 4 and c.859T→C in exon 8 (Ishihara, et al (2003)). The most common polymorphism in the 5' UTR of exon 1 was shown to cause increased PTEN expression when cloned and expressed in two cell lines. Exposure of the transfected cells to insulin showed a significant reduction in P-Akt/PKB compared to a control of wild-type PTEN, and this polymorphism may therefore contribute to insulin resistance in type-2 diabetes in this population.

4.2 Future Work

Although several compounds have been shown to positively affect PTEN mRNA expression including progesterone, TGF- β 1, and NGF, or protein levels, (vitamin D3 analogues, BDNF and BMP), few studies have proposed mechanisms which may underlie these observations. Additionally, few groups have analysed both mRNA and protein expression of PTEN in response to stimulation. It is not clear, therefore, how many studies in which increased PTEN mRNA was detected would also demonstrate a change in protein levels. As the regulation of PTEN may involve both transcriptional and post-translational events which may be further complicated by cell-type specificity, it is worthwhile investigating both levels of response for a complete picture. The fundamental question still remains as to how constitutive expression of PTEN is maintained, and how the compounds mentioned above actually affect changes in mRNA expression.

Recently, a study by Han, et al 2003 on mouse PTEN revealed a strong promoter in the 5' UTR at -551 to -220 upstream of the translation start codon. This promoter drives the production of an mRNA transcript with a shorter 5' UTR which was translated at 100-fold higher efficiency than the full-length 5' UTR transcript. The group also demonstrated constitutive activation of the promoter via Sp1. It would be of interest to determine whether this is also the case for the constitutive expression of human PTEN. For this purpose, the entire human 5'UTR upstream of exon 1 would be cloned into a reporter vector containing the luciferase gene, such as pGL-2-Basic (Promega). A range of clones with successively smaller portions of 5'UTR would also be created, and the clones transfected into a suitable mammalian

cell line. Degree of promoter activity would be assessed after 24–48 hours by fluorescence of cell extracts in the luciferase assay, in which luciferin is added to the extracts and light levels determined by scintillation counter. To assess the ability of Sp1 to initiate transcription, the transcription factor would be cloned into a *Drosophila* expression vector containing a strong promoter, such as pAC5.1 (Invitrogen) which is driven by the actin 5C promoter. Co-transfection of the Sp1 and PTEN 5'UTR clones into *Drosophila* SL2 cells (which contain no Sp factors) followed by the luciferase assay would be used to determine the degree of promoter activity in each PTEN clone. Clones exhibiting significantly higher levels of promoter activity would then be sequenced to determine whether one or more transcripts are present. As TGF- β 1 may affect PTEN transcription via Sp1, it would be interesting to stimulate co-transfected cells with the cytokine to ascertain whether treatment enhances promoter activity.

To further investigate the presence of nuclear PTEN in endometrial cell lines, the following approach could be taken. Firstly, transfected cells would be imaged using a confocal microscope with the ability to scan and create 3-D images to more accurately assess the location of PTEN-EGFP. Secondly, nuclear and cytoplasmic protein extracts would be prepared from transfected cells using a commercially available kit such as NE-PER (Pierce-Endogen). The two fractions would be used in Western blot and probed with an anti-PTEN antibody, with anti- β -actin as a control to demonstrate the purity of the nuclear fraction. As a lack of nuclear PTEN has been associated with some high-grade tumours, a cell line in which nuclear PTEN has been reported, such as MCF-7 breast cancer cells, would be used as a control.

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APPENDIX I

MEDIA AND BUFFER COMPOSITION

1. Cell Culture Media Composition

HEC-1B Cell Line

McCoy's 5A Modified Medium

For Complete Medium (CM) add the following to 500ml of McCoy's:

1% L-Glutamine

1% Penicillin-Streptomycin

10% Foetal Calf Serum

For Reduced-Serum Medium (RSM) add the following to 500ml McCoy's:

1% L-Glutamine

1% Penicillin-Streptomycin

1% Foetal Calf Serum

Ishikawa Cell Line

Minimal Essential Medium Eagle

For Complete medium (CM) add the following to 500ml of MEME:

1% L-Glutamine

1% Penicillin-Streptomycin

1% non-essential amino acids

5% Foetal Calf Serum

COS-7 Cell Line

Dulbecco's Modified Eagles Medium (DMEM) with Glutamax

For complete medium (CM) add the following to 500ml of DMEM :

1% Penicillin-Streptomycin

10% Foetal Calf Serum

2. Agarose Gel Electrophoresis

10x TAE Buffer:

Tris 32.3 g

EDTA 2.48 g

Glacial Acetic Acid 11.7 ml

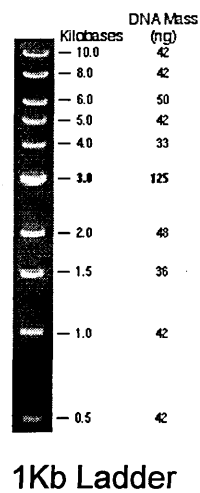
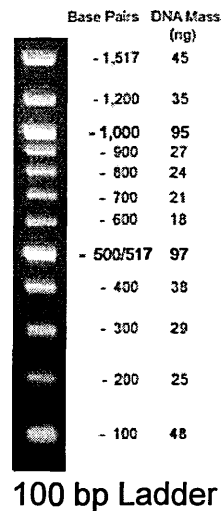
Make up to 1 L with distilled water.

Loading Buffer

Sucrose 25%

Xylene Cyanol FF 0.02%

Bromophenol Blue 0.05%



3. Protein Extraction

Activation of Sodium orthovanadate

Prepare a 200 mM solution of sodium orthovanadate. Adjust the pH to 10.0 to give a yellow solution. Boil the solution until it turns colorless and cool to room temperature. Readjust the pH to 10.0 and repeat the boiling and pH adjustment until the solution remains colorless and the pH stabilises at 10.0. Aliquot and store at -20°C .

Protein Lysis Buffer

| | |
|---|-------------------|
| Glycerol | 5ml |
| SDS | 0.5g |
| DTT | 0.5g |
| 1M Sodium fluoride | 5ml |
| 2M $\text{Na}_4\text{P}_2\text{O}_7$ | 0.5ml |
| 1M Na_3VO_4 (activated) | 0.05ml |
| 0.1M PMSF | 0.5ml |
| Protease inhibitor Cocktail | 300 μl |
| 1M Tris pH 6.8 | 3.125ml |

Add distilled water to 50ml, aliquot and store at -20°C .

4. SDS-PAGE & Western Blotting

Resolving Buffer

1.5M Tris/HCl pH 8.8

Stacking Buffer

0.5M Tris/HCl pH 6.8

10x Running Buffer

0.25M Tris/HCl

2M glycine

1% SDS (w/v)

Adjust to pH 8.3

Resolving Gel, 8% (2x minigels of 0.75mm thickness)

| | |
|------------------|-------|
| Resolving Buffer | 3.0ml |
|------------------|-------|

| | |
|------------|-------|
| Acrylamide | 1.6ml |
|------------|-------|

| | |
|-------------------|-------|
| dH ₂ O | 3.2ml |
|-------------------|-------|

| | |
|---------|------|
| 10% SDS | 80μl |
|---------|------|

Mix by inverting three times and add:

| | |
|---------|-------|
| 10% APS | 100μl |
|---------|-------|

| | |
|-------|------|
| TEMED | 40μl |
|-------|------|

Stacking gel, 4% (2x minigels of 0.75mm thickness)

| | |
|-----------------|--------|
| Stacking Buffer | 1.25ml |
|-----------------|--------|

| | |
|------------|-------|
| Acrylamide | 0.5ml |
|------------|-------|

| | |
|-------------------|-------|
| dH ₂ O | 3.1ml |
|-------------------|-------|

| | |
|---------|------|
| 10% SDS | 20μl |
|---------|------|

Mix by inverting three times and add:

| | |
|---------|-------|
| 10% APS | 100μl |
|---------|-------|

| | |
|-------|------|
| TEMED | 40μl |
|-------|------|

Towbin Buffer

Tris 4.84 g

Glycine 21.62 g

Methanol 150 ml

Dissolve in 1L of distilled water.

Chill for 2 hours at -20°C

10x TBS/T

20mM Tris

0.9% Sodium Chloride

Adjust the pH to 7.5 and make up to 1L with distilled water

Add 0.1% TWEEN-20

10x PBS/T

Sodium Chloride 80 g

Potassium Chloride 2 g

Disodium hydrogen orthophosphate 26.8 g

Potassium dihydrogen orthophosphate 2.4 g

Adjust the pH to 7.4 and make up to 1L with distilled water

Add 0.1% TWEEN-20

Coomassie Stain

Dissolve Coomassie R250 in destain to 0.25%

Store at 4°C .

Destain

Methanol 45ml

Acetic acid 5ml

Make up to 100ml with distilled water.

Protein Markers

| | 208KDa | 97.4KDa |
|---------------------------------|---------------|----------------------------|
| | 116 | |
| | 66 | 58.1 |
| | | 38.8 |
| | 45 | 29 |
| | 29 | 20.1 |
| | | 14.3 |
| High Range Colour Marker | | Biotinylated Marker |

5. Bacterial Culture

L-Broth

Tryptone 10g

Yeast Extract 5g

Sodium Chloride 5g

Add 900 ml of ultrapure water, adjust pH to 7.5, make up to a final volume of

1L

Autoclave to sterilise.

L-Agar Selective Plates & Stabs

Add kanamycin to cooled molten L-Agar to final concentration of 50µg/ml.

Pour 30ml per Petri dish and allow to set. For stabs pour 15ml into a sterile 50ml Falcon and allow to set at a 45° angle. Store at 4°C for up to 2 weeks.

RNase A Stock Solution

Dissolve 10 mg of RNase A in 1 ml of sterile water. Filter sterilise and store at -20°C.

6. Plasmid Isolation

Alkaline lysis solutions

Solution I

| | |
|---------|-------|
| Glucose | 50 mM |
|---------|-------|

| | |
|------|-------|
| EDTA | 10 mM |
|------|-------|

| | |
|-------------------|-------|
| Tris-HCl (pH 8.0) | 25 mM |
|-------------------|-------|

Add ultrapure water to a final volume of 100 ml.

Autoclave to sterilise and store at 4°C.

Solution II (Prepared fresh just prior to use):

| | |
|---------------------|--------|
| 2M Sodium Hydroxide | 100 µl |
|---------------------|--------|

| | |
|---------|--------|
| 10% SDS | 100 µl |
|---------|--------|

| | |
|-------|--------|
| Water | 800 µl |
|-------|--------|

Solution III

| | |
|----------------------|--------|
| 5M Potassium Acetate | 14.7 g |
|----------------------|--------|

Acetic Acid 5.75 ml

Add distilled water to 50 ml and store at 4°C.

7. Stimulation Stock Compounds

β-17-Estradiol (20µg/ml)

Dissolve the contents of the bottle in 1 ml of ethanol and add 49ml of McCoy's 5A medium. Store at 4°C.

TGFβ-1 (2µg/ml)

Dissolve the contents of the vial (2 µg) in 1 ml of sterile 4mM HCl containing 1% BSA. Store aliquots at -70°C.

Normal goat serum

Dilute in PBS to final concentration of 10% with 0.05% Sodium azide.

Aliquot and store at -20°C.

8. Extraction of Human DNA from Whole Blood

BufferA

Tris 1.2g

Sucrose 108.5g

Magnesium chloride 1g

Make up to 990ml with distilled water and adjust pH to 8.0.

Autoclave to sterilize. Add 10ml of TritonX-100 and store at 4°C.

Buffer B

| | |
|-----------------|--------|
| Tris | 48.44g |
| EDTA | 22.33g |
| Sodium chloride | 8.766g |

Make up to 900ml with distilled water and adjust pH to 8.0.

Autoclave to sterilize. Add 100ml of a 10% SDS solution and store at 4⁰C.

9. SSCP

Formamide Buffer

| | |
|-----------------------------|--------|
| Formamide | 9.5 ml |
| 10mM EDTA in 10mM NaOH | 0.5 ml |
| Blue / orange dye (Promega) | 200 µl |

10x TBE Buffer

| | |
|------------|------|
| Tris | 112g |
| Boric Acid | 55g |
| 1M EDTA | 20ml |

Make up to 1 L with distilled water.

SSCP Gel Mix, 80ml

0.5x MDE with 5% glycerol

glycerol

2x MDE 20ml

10x TBE 4.8ml

50% glycerol 8ml

Water 47.2ml

0.6x MDE with 10%

2x MDE 25ml

10x TBE 4.8ml

50% glycerol 16ml

Water 34.2ml

Mix by inverting three times.

Add 800 μ l of freshly prepared 25% Ammonium Persulphate and 80 μ l of TEMED.

Invert three times, pour between the glass plates and insert the teflon combs.

Manual Silver staining

Gel Fix

Ethanol 100ml

Glacial acetic acid 5ml

Add distilled water to 1L.

Silver stain

0.001% Silver nitrate in distilled water. Store in the dark.

Developer

5M Sodium hydroxide 30ml

Formaldehyde 1.5ml

Make up to 500ml with distilled water. Store in the dark.

Final fix

Sodium carbonate 3.75g

Make up to 500ml with distilled water.

APPENDIX II

PTEN Sequence Data

1. PTEN cDNA Sequence

```

1  ATGACA GCC ATC  ATC AAA GAG ATC GTT AGC  AGA AAC  AAA AGG AGA  15
16  TATCAA GAG GAT  GGA TTC  GAC TTA GAC TTG  ACC TAT  ATT  TAT  CCA  30
31  AACATT ATT GCT  ATG GGA TTT  CCT GCA GAA  AGA CTT  GAA GGC  GTA  45
46  TACAGG AAC AAT  ATT  GAT  GAT  GTA GTA AGG  TTT TTG  GAT  TCA AAG  60
61  CATAAA AAC CAT  TAC AAG ATA  TAC AAT  CTT  TGT GCT  GAA AGA  CAT  75
76  TAT  GAC ACC GCC AAA TTT AAT TGC AGA GTT  GCA CAA TAT  CCT TTT  90
91  GAA  GAC CAT AAC CCA CCA CAG CTA GAA CTT ATC AAA CCC TTT TGT 105
106 GAA  GAT CTT GAC CAA TGG CTA AGT  GAA  GAT  GAC AAT  CAT GTT  GCA 120
121 [GCA ATT  CAC TGT AAA GCT  GGA AAG  GGA CGA ACT  GGT  GTA ATG ATA] 135
136 TGT  GCA TAT TTA TTA CAT  CGG GGC AAA TTT  TTA AAG  GCA CAA GAG 150
151 GCC  CTA GAT TTC TAT GGG GAA GTA AGG ACC AGA  GAC AAA AAG  GGA 165
166 GTA  ACT ATTCCC AGT CAG  AGG CGC  TAT GTG  TAT TAT  TAT AGC  TAC 180
181 CTG  TTA AAGAAT  CAT CTG  GAT TAT  AGA CCA  GTG GCA  CTG TTG  TTT 195
196 CAC  AAG ATGATG  TTT GAA  ACT ATT  CCA ATG  TTC AGT  GGC GGA  ACT 210
211 TGC  AAT CCTCAG  TTT GTG  GTC TGC  CAG CTA  AAG GTG  AAG ATA  TAT 225
226 TCC  TCC AATTCA  GGA CCC  ACA CGA  CGG GAA  GAC AAG  TTC ATG  TAC 240
241 TTT  GAG TTC CCT  CAG CCG  TTA CCT  GTG TGT  GGT GAT  ATC AAA  GTA 255
256 GAG  TTC TTC CAC  AAA CAG  AAC AAG  ATG CTA  AAA AAG  GAC AAA  ATG 270
271 TTT  CAC TTT TGG  GTA AAT  ACA TTC  TTC ATA  CCA GGA  CCA GAG  GAA 285
286 ACC  TCA GAAAAA  GTA GAA  AAT GGA  AGT CTA  TGT GAT  CAA GAA  ATC 300
301 GAT  AGC ATTTGC  AGT ATA  GAG CGT  GCA GAT  AAT GAC  AAG GAA  TAT 315
316 CTA  GTA CTTACT  TTA ACA  AAA AAT  GAT CTT  GAC AAA  GCA AAT AAA 330
331 GAC  AAA GCCAAC  CGA TAC  TTT TCT  CCA AAT  TTT AAG  GTG AAG  CTG 345
346 TAC  TTC ACAAAA  ACA GTA  GAG GAG  CCG TCA  AAT CCA  GAG GCT  AGC 360
361 AGT  TCA ACTTCT  GTA ACA  CCA GAT  GTT AGT  GAC AAT  GAA CCT  GAT 375
376 CAT  TAT AGATAT  TCT GAC  ACC ACT  GAC TCT  GAT CCA  GAG AAT  GAA 390
391 CCT  TTT GATGAA  GAT CAG  CAT ACA  CAA ATT  ACA AAA  GTC TGA

```

The putative phosphatase catalytic domain in Exon 5 (boxed) includes the catalytic motif (red) at residues 122-133. N-terminal homology to tensin/auxilin is highlighted in grey. GenBank accession AF067844.

2. PTEN Genomic Sequences

Exons are highlighted in red and include a small portion of the adjacent intron, shown in black. Primer binding regions are shown highlighted in the text.

Exon 1 23338-23417bp

| | | | | |
|------------|------------|------------|------------|-------------|
| ccaccagcag | cttctgccat | ctctctcctc | ctttttcttc | agccacaggc |
| tcccagacat | gacagccatc | atcaaagaga | tcgttagcag | aaacaaaagg |
| agatatcaag | aggatggatt | cgacttagac | ttgacctgta | tccattttctg |
| cggctgctcc | tctttacctt | tctgtcactc | | |

Primer PTEN 1

Exon 2 51995-52079 bp

| | | | | |
|------------|------------|------------|-------------|------------|
| ttcagatatt | tctttcctta | actaaagtac | tcagatatatt | atccaaacat |
| tattgctatg | ggatttcctg | cagaaagact | tgaaggcgta | tacaggaaca |
| atattgatga | tgtagtaagg | taagaatgct | ttgattttct | atttcaaata |
| ttgatgttta | tattcatggt | gtgttttcat | | |

Exon 3 83481-83526 bp

| | | | | |
|------------|------------|-------------|------------|-------------|
| ttcttgatag | tattaatgta | atttcaaattg | ttagctcatt | tttggttaatg |
| gtggcttttt | gtttgtttgt | tttgtttttaa | ggtttttgga | ttcaaagcat |
| aaaaaccatt | acaagatata | caatctgtaa | gtatgttttc | ttatttgtat |
| gcttgcaaat | atcttctaaa | acaactatta | | |

Exon 4 89015-89058 bp

| | | | | |
|-------------|------------|-------------|------------|------------|
| ataactttat | atcactttta | aactttttctt | ttagttgtgc | tgaaagacat |
| tatgacaccg | ccaaatttaa | ttgcagaggt | aggtatgaat | gtactgtact |
| atgttggtata | acttaaacc | | | |

Exon 5 90987-91225 bp

| | | | | |
|------------|----------------------|-------------|------------|------------|
| tatgcaacat | ttctaaagtt | acctacttgt | taattaaaaa | ttcaagagtt |
| tttttttctt | attctgaggt | tatcttttta | ccacagttgc | acaatatcct |
| tttgaagacc | ataaccacc | acagctagaa | cttatcaaac | ccttttgtga |
| agatcttgac | c a a t g H H | | gtt | gcagcaattc |
| actgtaaagc | tggaaaggga | cgaactgggtg | taatgatatg | tgcatattta |
| ttacatcggg | gcaaattttt | aaaggcacia | gaggccctag | atttctatgg |
| ggaagtaagg | accagagaca | aaaaggtaag | ttattttttg | atgtttttcc |
| tttcctcttc | | | | |

Primers PTEN 4 PTEN 5A & 5B PTEN 5C & 5D

Exon 6 110086-110227 bp

| | | | | |
|------------|------------|------------|------------|------------|
| aacatttttt | ttcaatttgg | cttctctttt | ttttctgtcc | accagggagt |
| aactattccc | agtcagaggc | gctatgtgta | ttattatagc | tacctgttaa |
| agaatcatct | ggattataga | ccagtggcac | tggtgtttca | caagatgatg |
| tttgaaacta | ttccaatgtt | cagtggcgga | acttgcagta | agtgcttgaa |
| attctcatcc | ttccatgtat | tggaacagtt | ttcttaacca | |

Exon 7 115821-115987 bp

| | | | | |
|------------|------------|------------|------------|-------------|
| atcattaaaa | tcgtttttga | cagtttgaca | gttaaaggca | tttcctgtga |
| aataatactg | gtatgtattt | aaccatgcag | atcctcagtt | tgtgggtctgc |
| cagctaaagg | tgaagatata | ttcctccaat | tcaggaccca | cacgacggga |
| agacaagttc | atgtactttg | agttccctca | gccgttacct | gtgtgtgggtg |
| atatcaaagt | agagttcttc | cacaaacaga | acaagatgct | aaaaaagggt |
| tgtactttac | tttcattggg | agaaatatcc | aaaataagga | cagattaaaa |

Exon 8 118862-119086

| | | | | |
|------------|-------------|------------|-------------|------------|
| aaacatcatt | aattaaatat | gtcatttcat | ttctttttct | tttctttttt |
| ttttttttta | ggacaaaatg | tttactttt | gggtaaatac | attcttcata |
| ccaggaccag | aggaaacctc | agaaaaagta | gaaaatggaa | gtctatgtga |
| tcaagaaatc | gatagcattt | gcagtataga | gcgtgcagat | aatgacaagg |
| aatatctagt | acttacttta | acaaaaaatg | atcttoB B i | |
| | accgatactt | ttctccaaat | tttaagggtca | gttaaattaa |
| acattttgtg | gggggttggtg | acttgtatgt | atgtgatgtg | tgtttaattc |

Primers PTEN 8A & 8B PTEN 8C &**Exon 9 123258-123443 bp**

| | | | | |
|-------------|-------------|-------------|------------|------------|
| tgttaaaaat | atattttcact | aaatagttta | agatgagtca | tatttgtggg |
| ttttcatttt | aaattttctt | tctctagggtg | aagctgtact | tcacaaaaac |
| agtagaggag | ccgtcaaatac | cagaggctag | cagttcaact | tctgtaacac |
| cagatgttag | tgacaatgaa | cctgatcatt | atagatatct | tgacaccact |
| gactctgatac | cagagaatga | accttttgat | gaagatcagc | atacacaaat |
| tacaaaagtc | tgaatttttt | tttatcaaga | gggataaaac | accatgaaaa |

Primer PTEN 2a

APPENDIX III

ipPTEN/PTH2 cDNA Sequence

```

1  AGG  ACAGCC ATC ATC AAA GAG ATC GTT AGC AGA AAC AAA AGG AGA 15
16 TAT  CAAGAG GAT GGA TTC GAC TTA GAC TTG ACC TAT ATT TAT CTA 30
31 AAC  ATTATT GCT ATG GGA TTT CCT GCA GAA AGA CTT GAA GGC GTA 45
46 TAC  AGGAAC AAT ATT GAT GAT GTA GTA AGG TTT TTG GAT TCA AAG 60
61 CAT  AAAAAC CAT TAC AAG ATA CAC AAT CTT TGT GCT GAA AGA CAT 75
76 TAT  GAC ACC GCC AAA TCT AAT TAC AGA GTT GCG CAA TAT CCT TTT 90
91 GAA  GAC CAT AAC CCA CCA CAG CTA GAA CTT ATC AAA CCC TTT TGT 105
106 GAA  GAT CTT GAC CAA TGG CTA AGT GAA GAT GAC AAT CAT GTT GCA 120
121 GCA ATT CAC TGT AAA GCT GGA AAG GGA CGA ACT GGT ATA ATG ATT| 135
136 TGT  GCA TAT TTA TTA CAT CGG GGC AAA TTT TTA AAG GCA CAA GAG 150
151 |GCC CTA GAT TTC TAT GGG GAA GTA AGR ACC AGA GAC AAA AAG GGA| 165
166 GTA  ACT ATT CCC AGT CAG AGG cg! TAT GTG TAT TAC TAT AGC TAC 180
181 CTG  GTA AAG AAT CAT CTG GAT TAT AGA CCA GTG GCA CTG TTG TTT 195
196 CAC  AAG ATG ATG TTT GAA ACT ATT CCA ATG TTC AGT GGC GGA ACT 210
211 TGC  AAT CCT CAG TTT GTG GTC TGC CAG CTA AAG GTG AAG ATC TAT 225
226 TCC  TCC AAT TCA GGA CCC ACA CGA TGG GAG GAC AAG TTC ATG TAT 240
241 TTT  GAG TTC CCT CAG CCG TTA CCT GTG TGT GGT GAT ATC AAA GTA 255
256 GAG  TTC TTC CAC AAA CAG AAC AAG ATG CTA AAA AAG GAC AAA ATG 270
271 TTT  CAC TTT TGG GTA AAT ACA TTC TTC ATA CCA GGA CCA GAG GAA 285
286 ACC  TCA GAA AAA GTA GAA AAT GGA AGT CTA TGT GAT CAA GAA ATT 300
301 GAT  AGC ATT TGC AGT rATA GAG CGT GCA GAT AAT GAC AAG GAG TAT 315
316 CTA  GTA CTT ACT TTA ACA AAA AAT GAT CTT GAC AAA GCA AAT AAA 330
331 GAC  AAA GCC AAC CGA TAC TTT TCT CCA AAT GTT TSIG GTG AAG CTG 345
346 TAC  TTC ACA AAA ACA GTA GAG GAG CCG TCA AAT CCA GAG GCT AGC 360
361 AGT  TCA ACT TCT GTA ACA CCA GAT GTT AGT GAC AAT GAA CCT GAT 375
376 CAT  TAT AGA TAT TCT GAC ACC ACT GAC TCT GAT CCA GAG AAT GAA 390
391 CCT  TTT GAT GAA GAT CAG CAT ACA CAA ATT ACA AAA GTC TGA

```


The nucleotide sequence of 4JPTEN/PTH2 cDNA,. Putative phosphatase catalytic domain in Exon 5 (boxed), catalytic motif (red), N-terminal homology to tensin/auxilin (grey), and Putative Tyrosine Phosphorylation site (yellow). *M s e* I sites indicated by blue arrows, *H h s* I sites indicated by green arrows. Bases that differ from PTEN are indicated in bold and underlined. Taken from Dahia, *et al* 1998.

APPENDIX V

PCR PROGRAMMES

PTEN I Cycle

LID ON 110°C


| | | |
|------|------------|---|
| 95°C | 5 minutes | |
| 95°C | 1 minute |  |
| 55°C | 1 minute | |
| 72°C | 1 minute | |
| | | x 30 |
| 72°C | 10 minutes | |

LID OFF

4°C ∞

RIS 2.2 Cycle

LID ON 110°C


| | | |
|------|------------|---|
| 95°C | 5 minutes | |
| 95°C | 2 seconds |  |
| 50°C | 20 seconds | |
| 72°C | 20 seconds | |
| | | x 37 |
| 72°C | 10 minutes | |

LID OFF

4°C ∞

GAPDH Cycle

LID ON 110°C


| | | |
|------|------------|---|
| 95°C | 5 minutes | |
| 95°C | 1 minute |  |
| 58°C | 1 minute | |
| 72°C | 1 minute | |
| | | x 30 |
| 72°C | 10 minutes | |

LID OFF

4°C ∞

PAI-1 Cycle

LID ON 110°C

| | | |
|------|------------|---|
| 95°C | 5 minutes | |
| 95°C | 30 seconds |  |
| 60°C | 30 seconds | |
| 72°C | 30 seconds | |
| | | x30 |
| 72°C | 10 minutes | |

HEATED LID OFF

4°C ∞

Bcl-2 PCR Cycle

HEATED LID ON 110°C

95°C 5 minutes

95°C 30 seconds

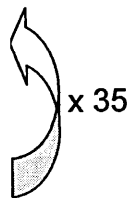
61°C 30 seconds

72°C 1 minute 30"

72°C 10 minutes

HEATED LID OFF

4°C ∞



Pfx3LX2 PCR Cycle

HEATED LID ON 110°C

95°C 2 minutes

95°C 1 minute

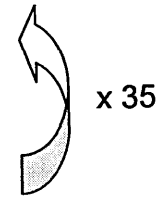
55°C 1 minute

68°C 1 minute

68°C 20 minutes

HEATED LID OFF

4°C ∞



APPENDIX VI

Equipment Suppliers

Mini-Gel Submarine Electrophoresis Units

Biometra Ltd, P.O. Box 42, Sale, Manchester.

Protean III Vertical Mini-Gel Electrophoresis Unit & Mini-Gel Electrophoresis
Power Pack 300.

BioRad Laboratories Ltd, Marylands Ave., Hemel Hempsted, Herts.

SSCP Gel Vertical Gel Apparatus Hoefer SE660 & 3000v Power Supply

Amersham Biosciences UK Ltd, Pollards Wood, Chalfont St. Giles,
Bucks.

PCR Primus Thermocyclers

MWG Biotech (UK) Ltd, Mill Court, Milton Keynes.

APPENDIX VII

| 1. Purity of RNA from Serum non-Starved HEC-1B Stimulated with TGF- β 1 | | | |
|---|-------|-----------------------|---------------|
| Concentration TGF- β 1 / Time (Hours) | Flask | Conc μ g/ μ l | Ratio 260/280 |
| 0ng/4hrs | A | 2.15 | 1.9 |
| | B | 1.64 | 1.7 |
| 0ng/8hrs | A | 1.79 | 1.8 |
| | B | 2.21 | 1.86 |
| 0ng/24hrs | A | 2.87 | 2.1 |
| | B | 2.99 | 1.78 |
| 2ng/4hrs | A | 1.3 | 1.9 |
| | B | 1.23 | 1.81 |
| 2ng/8hrs | A | 1.38 | 1.97 |
| | B | 1.35 | 2.01 |
| 2ng/24hrs | A | 1.44 | 1.89 |
| | B | 1.45 | 2.08 |
| 5ng/4hrs | A | 2.13 | 1.93 |
| | B | 1.67 | 1.86 |
| 5ng/8hrs | A | 1.4 | 1.9 |
| | B | 1.37 | 1.84 |
| 5ng/24hrs | A | 1.82 | 1.9 |
| | B | 2.05 | 1.92 |

| 2. Purity of RNA from Serum-Starved HEC-1B Stimulated with TGF-β1 Experiment 1 | | | |
|--|-------|-----------------------|---------------|
| Concentration TGF- β 1 / Time (Hours) | Flask | Conc μ g/ μ l | Ratio 260/280 |
| 10% Control | A | 1.64 | 1.65 |
| | B | 2.96 | 1.67 |
| 0ng/4hrs | A | 3.65 | 1.76 |
| | B | 5.68 | 1.9 |
| 0ng/8hrs | A | 3.26 | 1.9 |
| | B | 4.96 | 1.86 |
| 0ng/24hrs | A | 1.53 | 1.88 |
| | B | 2.1 | 1.98 |
| 2ng/4hrs | A | 5.42 | 1.8 |
| | B | 4.91 | 1.81 |
| 2ng/8hrs | A | 1.29 | 1.86 |
| | B | 5.45 | 1.79 |
| 2ng/24hrs | A | 6.82 | 1.84 |
| | B | 6.43 | 1.99 |
| 5ng/4hrs | A | 4.15 | 1.83 |
| | B | 5.26 | 1.84 |
| 5ng/8hrs | A | 1.72 | 1.75 |
| | B | 1.82 | 1.75 |
| 5ng/24hrs | A | 7.67 | 1.89 |
| | B | 6.76 | 1.92 |

3. Purity of RNA from Serum-Starved HEC-1B Stimulated with TGF- β 1 Experiment 2

| Concentration TGF- β 1 /Time (Hours) | Flask | Conc ug/ul | Ratio 260/280 |
|---|-------|------------|---------------|
| 10% Control | A | 1.168 | 1.79 |
| | B | 3.37 | 1.71 |
| 0ng/4hrs | A | 4.92 | 1.7 |
| | B | 3.24 | 1.56 |
| 0ng/8hrs | A | 4.49 | 1.7 |
| | B | 4.09 | 1.75 |
| 0ng/24hrs | A | 4.62 | 1.45 |
| | B | 6.1 | 1.73 |
| 2ng/4hrs | A | 3.56 | 1.5 |
| | B | 3.97 | 1.46 |
| 2ng/8hrs | A | 6.1 | 1.75 |
| | B | 7.83 | 1.78 |
| 2ng/24hrs | A | 7.68 | 1.74 |
| | B | 8.1 | 1.75 |
| 5ng/4hrs | A | 2.59 | 1.76 |
| | B | 6.94 | 1.68 |
| 5ng/8hrs | A | 2.98 | 1.6 |
| | B | 5.99 | 1.68 |
| 5ng/24hrs | A | 2.21 | 1.45 |
| | B | 6.11 | 1.65 |

4. Purity of RNA from Ishikawa cells Stimulated with TGF- β 1

| Concentration TGF- β 1 / Time (Hours) | Flask | Conc μ g/ μ l | Ratio 260/280 |
|--|-------|-----------------------|---------------|
| 0ng/4hrs | A | 2.8 | 1.66 |
| | B | 3.3 | 1.68 |
| 0ng/8hrs | A | 3.31 | 1.68 |
| | B | 2.96 | 1.69 |
| 0ng/24hrs | A | 4.13 | 1.66 |
| | B | 4.11 | 1.68 |
| 2ng/4hrs | A | 6.5 | 1.71 |
| | B | 3.6 | 1.73 |
| 2ng/8hrs | A | 3.2 | 1.72 |
| | B | 3.15 | 1.7 |
| 2ng/24hrs | A | 3.83 | 1.7 |
| | B | 3.04 | 1.68 |
| 5ng/4hrs | A | 4.3 | 1.69 |
| | B | 4.5 | 1.72 |
| 5ng/8hrs | A | 3.26 | 1.71 |
| | B | 3.2 | 1.66 |
| 5ng/24hrs | A | 3.52 | 1.69 |
| | B | 3.82 | 1.67 |

APPENDIX VIII

| Purity of RNA from HEC-1B Cell Density Experiment | | | |
|---|-------|-------------------|-------------------|
| Days in culture | Flask | Initial Density | |
| | | 4x10 ⁶ | 9x10 ⁶ |
| Day 1 | A | 1.85 | 1.88 |
| | B | 1.83 | 1.9 |
| | C | 1.88 | 1.89 |
| Day 2 | A | 1.77 | 1.8 |
| | B | 1.84 | 1.88 |
| | C | 1.8 | 1.81 |
| Day 3 | A | 1.8 | 1.82 |
| | B | 1.91 | 1.9 |
| | C | 1.8 | 1.8 |
| Day 4 | A | 1.85 | 1.88 |
| | B | 1.86 | 1.87 |
| | C | 1.85 | 1.89 |
| Day 4 MC | A | 1.87 | 1.89 |
| | B | 1.89 | 1.88 |
| | C | 1.79 | 1.84 |